Escherichia coli biofilm response to salicylic acid

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Abstract

In this study salicylic acid is proposed as an alternative biocide-free agent suitable for a preventive or integrative anti-biofilm approach. Indeed, salicylic acid has been proved to: i) reduce bacterial adhesion up to 68.1±5.6%; ii) affect biofilm structural development, reducing viable biomass by 97.0±0.7% and extracellular proteins and polysaccharides by 83.9±2.5% and 49.5±5.5% respectively, iii) promote biofilm detachment 3.4±0.6 fold. Moreover, salicylic acid treated biofilm has shown an increased amount of intracellular (2.3±0.2 fold) and extracellular (2.1±0.3 fold) reactive oxygen species, and resulted in increased production of the quorum sensing signal indole (7.6±1.4 fold). For the first time, experiments have revealed that salicylic acid interacts with proteins that play a role in quorum sensing, reactive oxygen species accumulation, motility, extracellular polymeric matrix components, transport and metabolism.

Keyword: salicylic acid, biofilm, anti-biofilm strategies, salicylates

Introduction

Biofilms, complex surface-associated communities of microorganisms embedded in a self-produced polymeric matrix, are up to several orders of magnitude more resistant to antimicrobial agents than their planktonic counterpart (Villa et al. 2010; Cappitelli et al. 2014; Polo et al. 2014). As a consequence, chemical treatment to prevent biological damage often involves considerable amounts of dangerous substances (Villa et al. 2012b). Today's emphasis is on approaches that exploit the potential of natural products to deprive microorganisms of their ability to develop biofilm, in a non-toxic way and with modalities that decrease the selection pressure for drug-resistant mutations (Cattò et al. 2015). Interference with the key step orchestrating biofilm formation is considered a promising strategy for developing innovative preventive anti-biofilm products. Indeed, microorganism adhesion, biofilm maturation as well as detachment have emerged as good points of attack (Cattò et al. 2015).

Salicylic acid (SA) is a secondary metabolite widely distributed throughout the plant kingdom (Vlot et al. 2009). It is involved in several physiological processes including the regulation of seed germination, stomatal closure, ion uptake through roots, the stimulation of flowering and response to abiotic stresses (Raskin 1992). More importantly, it is a signal molecule involved in the sophisticated ecological strategy that plants have adaptively developed to prevent harmful bacterial colonization on their living tissue, in response to an ever-present pathogen pressure (Faber & Wolff 1993; Lagonenko et al 2013). The large amount of available information concerning the lack of toxicity to human health, and safe behaviour in the environment, has suggested the potential of a SA-based anti-biofilm strategy without toxicity and safety concerns. Indeed, SA has been observed to reduce biofilm formation in both human and plant pathogens including *Bacillus cereus* (Lemos et al. 2014), *Pseudomonas fluorescens* (Lemos et al. 2014), *P. aeruginosa* (Prithiviraj et al. 2005; Chow et al. 2011), *Staphylococcus epidermidis* (Teichberg et al. 1993; Muller et al. 1998), *Salmonella enterica* (Rosenberg et al. 2008) and various microorganisms on catheters (Faber et al., 1993; El-Banna et al. 2012). However, the majority of these studies has been limited to the early step of biofilm formation (ie, adhesion step), as largely performed by simple systems based on static and batch-growth conditions that scarcely reproduce the complexity of biofilms in the real environments (Merrit et al. 2005). To the best of our knowledge, only few papers have investigated the SA anti-biofilm effects on a mature and well-developed biofilm typic of microbial systems. Moreover, the SA anti-biofilm mechanism of action, the nature of its binding targets and cellular receptors remain unknown (Damman 2013; Kumar 2014). In this study, an high-level and realistic approach has been proposed to greatly advance the current state of art about the behaviour of SA and its interaction with biofilm. Indeed, the ability of SA to prevent biofilm formation by interfering with bacterial adhesion, biofilm structural development and biofilm dispersal was thoroughly investigated by setting up an *Escherichia coli* lab-scale model system able to simulate conditions encountered in-vivo. In addition, the ability of SA to interfere with oxidative stress and with indole

77 signalling was explored. Moreover, for the first time, the putative interacting targets of SA were
78 isolated and identified among a complex mixture of *E. coli* proteins. Two SA derivatives have been
79 preliminary tested for their anti-adhesion performance.
80
81 **Materials and Methods**
82 ***Compounds***
83 SA, salicylic acid sodium salt (Na-SA) and 4-amino salicylic acid (4-ASA) (purity ≥99.0%) were
84 purchased by Sigma-Aldrich and were used without any further purification. 4-acetamidosalicylic
85 acid (4-AcASA) was synthesized starting from commercially available 4-ASA following a standard
86 procedure reported in the supplementary material.
87
88 ***E. coli strain and growth condition***
89 The well characterized *E. coli* K-12 wild-type strain ATCC 25404 was used as a model system for
90 bacterial biofilm. The strain was stored at –80°C in suspensions containing 20% glycerol and 2%
91 peptone, and was routinely grown in Luria-Bertani broth (LB, Sigma-Aldrich) at 30°C for 16 h.
92 Cells were washed three times with phosphate buffered saline (PBS, 0.01 M phosphate buffer,
93 0.0027 M potassium chloride pH 7.4, Sigma-Aldrich) and quantified with Thoma counting assay
94 prior to being used in the following experiments.
95
96 ***E. coli planktonic growth***
97 The ability of bacteria to grow with SA as the sole carbon and energy source was tested according
98 to Cattò et al. (2015). Briefly, a mineral medium (KH_2PO_4 30 g l^{-1}, Na_2HPO_4 70 g l^{-1}, NH_4Cl 10
99 g l^{-1}, pH 7) was prepared with the addition of 0 (negative control), 0.183, 1.83, 18.3, 183 and 1,830
100 µM SA and 3% of dimethyl sulphoxyde (DMSO) to make the molecule soluble. Bacteria of a
101 washed overnight culture were added to a final concentration of 10^7 cells ml^{-1} and grown at 30°C

for 72 h. At the end of the experiment, the absorbance at 600 nm (A_{600}) was measured by an UV/VIS 7315 Spectrophotometer (Jenway). The SA concentrations chosen were in line with a previous work, by Cattò et al. (2015), about the screening of different potential anti-biofilm compounds. The positive control was the mineral medium supplemented with glucose at both 1,830 µM and 3,000 µM.

Minimal inhibitory concentration (MIC) of SA was determined according to Cattò et al. (2015). Briefly, planktonic growth assays were carried out in LB medium supplemented with 0 (negative control), 0.183, 1.83, 18.3, 183 and 1,830 µM SA and 3% DMSO in 384-well microtiter plates. Bacteria of a washed overnight culture were added to a final concentration of 10^7 cells ml^{-1} and grown at 30°C. Growth was followed by measuring the absorbance at A_{600} every 10 min for over 24 h using the Infinite 200 PRO Microplate Reader (Tecan). Absorbance-based growth kinetics were constructed by plotting the A_{600} of suspensions minus the A_{600} of the non-inoculated medium against incubation time. The polynomial Gompertz model was used to fit the growth curves, and the maximum specific growth rate (A_{600} min^{-1}) and lag time (min) were calculated using GraphPad Prism software (version 5.0, San Diego, CA, USA). MIC corresponds to the concentration at which the lag time or the maximum specific growth rate was statistically significantly inferior to the negative control. Obtained data were normalized to the area, and means were reported. No anti-microbial activity was detected by the addition of DMSO.

The pH value of each medium supplemented with SA was measured using a Jenway 3510 pH Meter.

Three biological replicates were performed for each treatment and six technical replicates were performed for each experiment.

Cell adhesion

Cell adhesion was assessed in hydrophobic black-sided plates as previously reported by Cattò et al. (2015). Briefly, 200 µl of PBS containing 10^7 cells of a washed overnight culture supplemented with 0 (negative control), 0.183, 1.83, 18.3 and 183 µM of SA and 3% of DMSO were placed in microtiter plate wells. Cells were incubated for 18 h at 30°C. The microtiter plate wells were washed twice with 200 µl PBS, and adhered cells were stained using 10 µg ml^{-1} 4,6-diamidino-2-phenylindole (Sigma-Aldrich) in PBS for 20 min in the dark at room temperature. Fluorescence intensity was measured using the Infinite 200 PRO Microplate Reader (Tecan) at excitation wavelength of 335 nm and emission wavelength of 433 nm. A standard curve of fluorescence intensity versus cell number was determined and used to quantify the number of adhered cells in response to SA. Obtained data were normalized to the area and means were reported. No anti-adhesion activity was detected by the addition of DMSO.

Percentage reduction in comparison to the negative control was also calculated as: (SA data – negative control data) × 100 / negative control data. SA concentrations able to reduce the number of *E. coli* adhered cells by less than 20%, with respect to the negative control, were considered to be without anti-adhesion activity, between 20% and 30% with low anti-adhesion activity, between 30% and 40% with moderate anti-adhesion activity, and more than 40% with excellent anti-adhesion activity (Cattò et al. 2015).

Three biological replicates were performed for each treatment and six technical replicates were performed for each experiment.

Biofilm structural development

E. coli biofilm growth

E. coli biofilm without and with 183 µM SA was grown in the Centre for Disease Control Bioreactor (CDC reactor, Biosurface Technologies, Bozeman, MT, USA). SA-Na was used to increase SA solubility, sidestepping the addition of DMSO, with the perspective of a real

application in a solvent-free field. The inoculum of the bioreactor was prepared by inoculating 400 ml of sterile LB medium without and with SA (pH 7.3) with 1 ml of diluted overnight culture containing 10^7 cells of *E. coli* strain and growing this culture at 30°C under continuous stirring for 24 h. After the 24-h adhesion phase, the peristaltic pump was started and sterile 10% LB medium (pH 7.3) without and with SA was continuously pumped into the reactor at a rate of 8.3 ml min^{-1}. After 48 h of dynamic phase, the coupons were removed and gently washed with PBS. The obtained biofilms, grown without and with 183 μM SA, were analysed for their cell abundance, extracellular polymeric substances (EPS) composition, indole production and reactive oxygen species (ROS) content.

Cell abundance

The collected coupons were transferred to 5 ml PBS and biofilm was removed from the coupon surface by 1 min vortex mixing, 2 min sonication (50% amplitude, in water-bath; Branson 3510, Branson Ultrasonic Corporation, Dunburry, CT) followed by another 1 min vortex mixing. To remove bacterial aggregates, cell suspensions were homogenized by two 30 s cycle at 14,500 rpm (T 10 basic Ultra-Turrax) followed by 30 s vortex mixing. Viable cells in the biofilm were quantified by plating the cell suspension in LB agar medium. Obtained data were normalized to the area, and means were reported. SA performance was calculated as percentage reduction in sessile cells with respect to the control sample. The percentage of live and dead cells in the biofilm biomass was also calculated using the Live/Dead BacLight viability kit (Molecular Probes-Life Technologies) according to kit instructions. Fluorescence intensity was measured using the Infinite 200 PRO Microplate Reader (Tecan) with excitation at 480 nm and emission at 516 nm for the live green cells, and excitation at 581 nm and emission at 644 nm for the red dead cells. A standard curve of fluorescence intensity versus cell number was determined and used to quantify the number of live and dead cells. Relative viability within the biofilms was also determined by dividing the

percent of live cells by the percent of the dead cells in each sample. Three biological replicates were performed for each treatment and six technical replicates were performed for each experiment.

EPS composition

Collected coupons were transferred to 2% ethylenediaminetetraacetic acid (EDTA) and biofilm was removed from the coupon surface by vortex mixing and sonication followed by homogenization, as reported above for the cell abundance analysis. EPS extraction was performed as described by Villa et al. (2012a). The Bradford method (1976) was applied for analysing protein concentrations, whereas the phenol-sulfuric acid assay was applied for polysaccharides determination (Masuko et al. 2005) using glucose as the standard. Absorbance was measured using a UV/VIS 7315 Spectrophotometer (Jenway). Obtained data were normalized to the number of adhered cells, divided for the area, and means were reported. Three biological replicates were performed for each treatment and six technical replicates were performed for each experiment.

Biofilm Imaging by Confocal Laser Scanning Microscopy (CLSM)

Three-D morphology of biofilm without and with 183 μM SA was analysed by CLSM. Biofilm was stained with the lectin Concanavalin A-Texas Red conjugate dye (C825, Molecular Probes-Life Technologies) to visualize the EPS polysaccharide component and Sybr green I fluorescent nucleic acid dye (S7563, Molecular Probes-Life Technologies) to display biofilm cells. Biofilm was incubated with 200 μg/mL of ConA and 1:1000 of commercial Sybr green I dye solution in PBS at room temperature in the dark for 30 min and then rinsed with PBS. Coupons without biofilm were also stained in order to exclude any false positive signals. Biofilm samples were visualized using a Leica SP5 CLSM with excitation at 488 nm, and emission <530 nm (green and red channel). Images were captured with a 63×, 0.9 NA water immersion objective and analysed with the software Imaris (Bitplane Scientific Software, Zurich, Switzerland).

200 ***Biofilm dispersion***

201 After 48 h of dynamic phase, 5 ml of the output waste medium were collected and the amount of
202 dispersed cells was quantified by A_{600} density measurements (UV/VIS 7315 Spectrophotometer;
203 Jenway). Obtained data were normalized to the negative control and means were reported. Three
204 biological replicates were performed for each treatment and six technical replicates were performed
205 for each experiment.
206 In order to study the strength of biofilm to surface detachment after a SA treatment, coupons with
207 biofilm pre-grown without and with 183 μM SA were removed from the CDC reactor and soaked in
208 PBS for 24 h. At the end of experiment, biofilm was dislodged from the coupon surface as reported
209 in the previous section and viable cells were quantified by plate count viability assay. Cells in the
210 bulk PBS were also collected and quantified by plate count viability assay. The tendency of biofilm
211 to surface detachment was calculated as: (no. of viable cells from bulk PBS × 100)/(no. of viable
212 cells from bulk PBS + no. of viable cells from the coupon biofilm) and means were reported. Three
213 biological replicates were performed for each treatment and six technical replicates were performed
214 for each experiment.
215
216 ***Level of oxidative stress***

217 Collected coupons were transferred to 5 ml of 50 mM PBS and biofilm was removed from the
218 coupon surface by vortex mixing and sonication followed by homogenization as reported above for
219 the cell abundance analysis. Biofilm was centrifuged at 12,000 g at room temperature for 25 min.
220 The supernatant was filtered through 0.2 μm polycarbonate membranes and analysed for
221 extracellular ROS content. The pellet containing the cellular fraction was washed with 50 mM PBS,
222 suspended in 1 ml of 50 mM PBS and cells were broken by sonication (seven 1-min sonication
223 cycles at 22 μm amplitude followed by 2-min cooling periods, in Soniprep 150) in order to be
224 analysed for the intracellular ROS content. The level of extracellular and intracellular oxidative

stress was determined using the 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) assay according to Jakubowski and colleagues (2000). Briefly, 5 mM H_2DCFDA was added to the sample to a final concentration of 10 mM and samples were incubated at 30 °C. After 30 min, the samples were clarified by centrifugation and fluorescence of the supernatant was measured using the Infinite 200 PRO Microplate Reader (Tecan) with excitation at 488 nm and emission at 520 nm. Fluorescence values were normalized to the number of cells and means were reported. Three biological replicates were performed for each treatment and six technical replicates were performed for each experiment.

Indole signalling

Indole production

The collected coupons were transferred to 5 ml PBS and biofilm was removed from the coupon surface by vortex mixing and sonication followed by homogenization as reported above for the cell abundance analysis. Biofilm was centrifuged at 12,000 g at room temperature for 25 min. The supernatant as well as the pellet were set up as reported in the ROS assay using PBS. Five ml of the output waste medium released by the CDC reactor after 48 h of dynamic phase were also collected, filtered through 0.2 µm polycarbonate membranes and analysed for their indole content. The level of intracellular and extracellular indole as well as the indole concentration released in the output waste medium was investigated as described by Kuczyńska-Wiśnik and colleagues (2010). Briefly, Kovac's reagent (50 g l^{-1} 4-(dimethylamino)benzaldehyde, 710 g l^{-1} isoamylic alcohol, 240 g l^{-1} hydrochloric Acid) was mixed with the sample in the ratio 2:5 and after 2 min the absorbance at 540 nm was measured using a UV/VIS 7315 Spectrophotometer (Jenway). The indole concentration was calculated based on a calibration curve, and data were normalized to the number of cells and reported as the means of this data. Three biological replicates were performed for each treatment and three technical replicates were performed for each experiment.

In silico binding of SA to TnaA

The SA binding site to TnaA, the main protein involved in the indole production (Hu et al. 2010), was initially localized by means of blind docking calculations. Then, in order to acquire more accurate details about the SA binding mode in the previously recognized binding site, additional docking calculations were performed, limiting the binding site in the protein area suggested in the first blind docking attempt.

The SA structure was created, and structurally optimized using Gaussian09 package (Frisch et al. 2009), at the DFT/B3LYP/6-31G(d) level of theory. The carboxyl group was considered in the ionized form for consistency with the expected protonation state at physiological pH.

The computational model of the TnaA utilized for theoretical studies was retrieved from the Protein Data Bank (PDB accession code 2C44) (Ku et al. 2006). Despite the low resolution (2.8 Å), this X-ray structure contains the full length sequence and the complete structure of the tetramer. Prior to starting docking calculations, the sulphate and potassium ions present in the X-ray structure were removed. Moreover, the side chains of some residues and the hydrogen atoms had to be added by *tleap* module of Amber12 (Case et al. 2012). Then, to predict the most probable SA binding site, blind docking calculations were performed by the Autodock4/Vina package (Trott & Olson 2010), using the first subunit of TnaA (chain A). Then, 100 poses were generated and sorted according to the estimated SA binding free energy (ΔG).

The pocket in which SA showed the lowest ΔG was the target of an additional docking run performed by GOLD 5.2.2 (Hartshorn et al. 2007), to better refine the binding mode previously supposed for SA. On this occasion, 200 docking poses were generated and the ChemPLP scoring function was applied.

Figures were generated by PyMOL Molecular Graphics System, version 1.6, Schrodinger, LLC (http://www.pymol.org).

Isolation of the SA putative target protein

Isolation of the putative SA interactive proteins was carried out by a pull-down system combined with a mass spectrometry-based approach as described in Cattò et al. (2015).

E. coli planktonic cells were pelleted by centrifugation at 12,000 g at room temperature for 25 min, suspended in a protein extraction buffer (200 mM Tris-HCl, 40 mM NaCl, pH 7.5), and a soluble protein extract was obtained by cell sonication as reported in the level of oxidative stress section (Soniprep 150).

A NHS-Activated Sepharose 4 Fast Flow (GE Healthcare Life Sciences) was used as solid phase and 4-ASA was used as the ligand reagent in the preparation of the functionalized matrix (4-ASA/matrix) as reported by Cattò et al. (2015). Matrix hydrolysates were analysed by a liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) on an Ultimate 3000 Micro HPLC apparatus (Dionex) equipped with a FLM-3000-Flow manager module directly coupled to a LTQ Orbitrap XL hybrid FT mass spectrometer (Thermo Fisher Scientific). Reverse-phase chromatography was performed on a Jupiter C18, 5 μm, 150 x 1.0 mm column (Phenomenex). Mass spectra were collected at 60,000 resolutions in the Orbitrap analyser (mass range 50–1,000 m/z) in positive ion mode. High resolution MS data were elaborated manually using the Xcalibur Qual Browser software (version 2.2, Thermo Fisher Scientific).

The 4-ASA/matrix was incubated with the freshly-prepared soluble protein extract for 2.5 h at room temperature in a rotary shaker. The incubation mixture was then packed into a 15 ml chromatography column (i. d. 10 mm), washed to remove unbound proteins, and proteins specifically bound to the functionalized matrix were recovered by competitive elution with SA. Proteins in the recovered fraction were quantified by the Bradford method (1976) and submitted to electrophoretic analysis (SDS-PAGE) according to Laemmli et al. (1970). Coomassie-stained bands were manually excised from gels and submitted to trypsin digestion as previously described by Di Pasqua et al. (2010). Trypsin digested peptides were analysed by LC-ESI-MS using the same

300 experimental condition for the matrix hydrolysed analysis. Protein identification was made by the
301 software Proteome Discoverer (version 1.4, Thermo Fisher Scientific) after searching a
302 UniProtKB/Swiss-Prot Protein Knowledgebase [release 2013_12 of 11-Dec-13; taxonomical
303 restriction: *E. coli* (strain K12)] as already described by Cattò et al. (2015) with more stringent
304 thresholds (minimum protein score of 50 and on a decoy database search calculated false discovery
305 rate under 1%).
306 A NCBI-BLASTP (BLASTP 2.4.0) search against all non-redundant databases of Bacteria was also
307 performed using each of the identified proteins as a query. Returned protein sequences showing an
308 identity <30% and an expect-value threshold $>10^{-5}$ were discarded, being considered not
309 significantly similar to the query sequence (Pearson 2013). The bit score value in the BLASTP
310 output was used as a measure of sequence similarity (Pearson 2013).
311
312 ***Planktonic grow, cell adhesion and TnaA interaction of 4-ASA and 4-AcASA***
313 The ability of *E. coli* to grow with 4-ASA and 4-AcASA as the sole carbon and energy source and
314 their MICs were performed according to the protocol reported in the section '*E. coli planktonic*
315 *growth'*. The anti-adhesion performance of 4-ASA and 4-AcASA was determined according to the
316 procedure described in the '*Cell adhesion*' section. Moreover, the 4-ASA and 4-AcASA interaction
317 with TnaA was predicted by docking calculations. The applied computational procedure was
318 analogous to the one applied for SA, previously described.
319
320 ***Statistical analysis***
321 Two-tailed ANOVA and Student's *t*-test analysis, via a software run in MATLAB environment
322 (Version 7.0, The MathWorks Inc, Natick, USA), were applied to statistically evaluate any
323 significant differences among the samples and concentrations. ANOVA and Student's *t*-test analysis
324 were carried out after verifying data independence (Pearson's Chi-square test), normal distribution

(D'Agostino-Pearson normality test) and homogeneity of variance (Bartlett's test). Tukey's honestly significant different test (HSD) was used for pairwise comparison to determine the significance of the data. Statistically significant results were depicted by *p*-values ≤0.001.

Results

SA does not affect E. coli planktonic growth

With the aim of preventing biofilm formation, the ability of potential anti-biofilm compounds to be a nutrient source must be excluded (Cattò et al. 2015). The experiment revealed that *E. coli* did not grow in the mineral medium supplemented with SA as the sole carbon and energy source, at all tested concentrations (Table 1).

Figure 1a shows the planktonic growth results obtained without and with different concentrations of SA in LB medium. Indeed, there was a significant reduction in the maximum specific growth rate of *E. coli* at the maximum concentration tested, compared to the negative control (Figure 1b). The Minimal Inhibitory Concentration (MIC) assay results indicate that SA concentrations ≤183 μM had no effect on bacterial growth, while concentrations ≥1,830 μM inhibited *E. coli* growth. Therefore, concentrations ≤183 μM SA were considered sub-lethal and used in the subsequent studies.

SA inhibits E. coli cell adhesion

The anti-adhesion performance of SA was assessed by quantifying the number of adhered cells with respect to the negative control (Table 2). According to the anti-biofilm ranges proposed by Cattò et al. (2015), SA displayed low anti-adhesion activity at 0.183 and 1.83 μM, moderate anti-adhesion activity at 18.3 μM and excellent anti-adhesion activity at 183 μM, reducing, at this concentration, the number of adhered cells with respect to the negative control by up to 68.1±5.6%. Therefore, 183

μM was chosen as the best sub-lethal concentration, and it was used in the subsequent studies to further investigate the *E. coli* biofilm response to SA.

SA affects E. coli biofilm structural development

SA activity against *E. coli* biofilm development was studied using a lab-scale model system able to simulate condition encountered in vivo. A CDC reactor was used to grown a complex and mature *E. coli* biofilm without and with 183 μM SA.

Experiments revealed a biofilm that was seriously damaged when treated with 183 μM SA. Indeed, plate count viability assay showed that SA significantly reduced the number of viable cells adhered on the coupon surface by 97.0±0.7% with respect to the negative control (Figure 2a). Accordingly, the live/dead assay revealed a reduction of 97.4 ±12.9% in the number of live cells when biofilm was treated with SA in comparison to the untreated one. Moreover, no significant differences in the relative viability were found between the control and the treated biofilm, confirming that 183 μM SA reduces biofilm biomass with a mechanism that does not affect bacterial vitality(Figure 2b).

The EPS composition of biofilm grown without and with 183 μM SA in the CDC reactor was also investigated. The results showed a significant reduction in the amount of both the protein (83.9±2.5%) and the polysaccharide (49.5±5.5%) in SA-treated biofilm, compared to the untreated sample (Figure 2c).

In line with the previous results, side views of 3D reconstructed CLSM images of control biofilm depicted an intense fluorescence signal corresponding to multi-layers of cells (green signal) organized in macro-colonies inside a well-structured polysaccharide matrix (red signal) (Figure 2d). On the contrary, biofilm treated with SA displayed a significant decrease in thickness with a mono-layer of dispersed cells and a significant lower amount of polysaccharide matrix (Figure 2e).

SA is a biofilm dispersing agent

Density measurements of the waste medium after 48 h of dynamic phase were performed to quantify the amount of dispersed cells from a biofilm grown without and with 183 μM SA. The obtained data revealed that SA significantly increased the number of dispersed cells in the output waste medium by 78.0±0.6%, suggesting its possible role as a biofilm dispersing agent (Figure 3a). In order to study the strength of biofilm to surface detachment after SA treatment, a further experiment was carried out in which biofilm, pre-grown without and with SA, was soaked in PBS. After 24 h the number of viable cells remaining on the coupon surface and those released in the bulk liquid were quantified. The tendency of biofilm towards surface detachment was 3.4±0.6 fold higher in the SA-treated biofilm, confirming that SA makes biofilm more prone to detach from the surface (Figure 3b).

SA affects oxidative stress

The level of ROS within biofilm grown without and with 183 μM SA was also investigated. Our data show that biofilm exposed to SA is more prone to accumulate ROS, with a significant increase in both intracellular (2.3±0.2 fold) and extracellular (2.1±0.3 fold) ROS in SA treated biofilm, compared to the control (Figure 4).

SA interferes with the quorum sensing signal indole

Taking into account the important role played by quorum sensing signals in biofilm structural development and dispersion (Hu et al. 2010), the intracellular and extracellular amount of the quorum sensing signal indole was quantified in biofilm grown in the CDC reactor without and with 183 μM SA. The experiment revealed a significant increase in the amount of extracellular indole (78.3±16.6%) in biofilm grown in the presence of SA, compared to the control sample, whereas the intracellular indole concentration was comparable in both experiments (Figure 5a). Moreover, considering that the flow conditions would disperse the indole produced by biofilm, the indole

399 amount released by SA-treated biofilm into the output waste medium was calculated and compared
400 to the untreated sample. The obtained data show that biofilm treated with SA released into the waste
401 medium a significantly greater amount of indole (7.6±1.4 folds) than did the control sample (Figure
402 5a).
403 As tryptophanase (TnaA) is the main protein responsible for indole production, the hypothetical
404 mechanism by which SA could interact with TnaA was investigated by computational techniques.
405 Blind docking calculations suggested that SA could be bound to the most positively charged
406 enzyme area (blue area in Figure 5b) created by the side chains of the residues Arg419, Arg230,
407 Lys270 and Asn198. Refinement docking calculations confirmed that the carboxyl group of SA is
408 able to create a hydrogen-bond assisted salt bridge with the side chains of Lys270, Arg230 and
409 Arg419 (Figure 5c). An additional hydrogen bond could be formed by the same group of SA and
410 the side chain of Asn198, while the SA hydroxyl group could be involved in an additional hydrogen
411 bond with the side chain of Thr52 (Figure 5c). Interestingly, the superimposition of the SA/TnaA
412 complex with the X-ray structure of the enzyme reported by Ku et al. (2006) (PDB code 2C44)
413 showed that the polar groups of SA created the same hydrogen-bond network produced by one of
414 the sulphate ions found in the tryptophanase X-ray structure (Figure 5d). In fact, it is well accepted
415 that sulphates occupy the binding site physiologically occupied by the carboxyl group of the
416 tryptophan. Furthermore, interaction of SA with Lys270, covalently bound with pyridoxal
417 phosphate (PLP), was noted. For this reason, in order to verify if the binding of SA could be
418 influenced by the presence of PLP, two sessions of docking calculations were again performed
419 utilizing as the target the recently reported X-ray crystal structure of tryptophanase in the *holo* form
420 (PDB code 4W4H) (Kogan et al. 2015). Remarkably, we attained similar results, suggesting that SA
421 binding is not affected by the presence of PLP.
422
423 ***The salicylate group interacts with some E. coli proteins***

Assuming that primary events of anti-biofilm activity are triggered by interaction with a protein, the whole *E. coli* soluble proteome was screened to evaluate the functional ability of SA in targeting a protein. The 4-aminosalicylic acid (4-ASA) was chosen as the suitable molecule to successfully immobilize the SA scaffold on the solid matrix via an amide bond (4-ASA/matrix).

To verify the presence of the grafted salicylate group, the 4-ASA/matrix was hydrolysed and an in-depth analysis of the hydrolysates was carried out by mass spectrometric techniques. Mass spectrometry analysis registered a main monoisotopic single charged mass ([M+H]+) of 154.049 *m/z* in the hydrolysates produced by 4-ASA/matrix (Figure 6a). The theoretical [M+H]+ for 4-ASA is reported to be 154.050 *m/z*. There is significant evidence that 4-ASA was the main product released during hydrolysis (experimental mass error of 0.001 Da) and consequently 4-ASA/matrix was successfully functionalized with the salicylate group bound in the *para* position via the hydrolysable amide.

E. coli proteins bound to the 4-ASA/matrix were eluted by competition with SA, and collected fractions were analysed by SDS-PAGE. A number of bands were clearly observable in the SDS-PAGE profiles of the fractions eluted in the presence of SA (Figure 6b). Bands were also still evident in fractions collected after the main elution fraction, suggesting a lowered elution migration for some proteins. No bands were eluted when the control matrix (EA/matrix) replaced 4-ASA/matrix in the pull-down experiments. SDS-PAGE bands co-migrating with proteins pulled-down from the 4-ASA/matrix in independent experiments were selected and submitted to mass spectrometric analysis for protein identification. The identified proteins are reported in Table 3. Proteins targeted by SA were submitted to a BLAST search against all non-redundant databases of Bacteria and their similarity with protein sequences in selected pathogenic bacteria is reported in Figure 7. The BLAST search showed that the proteins targeted by SA are widespread in a variety of *E. coli* strains, as well as in other gram negative and gram positive bacteria. Indeed, GroL, GlnA, Tdh, FabZ and HldD were conserved in all the selected pathogenic bacteria (bit score always >80

449 for GroL, GlnA, Tdh and FabZ). Note that FkpA, WrbA, FklB and SecB were present in all gram
450 negative strains, while TnaA, FtnA, SbmC and MenI were conserved in an equal manner in both
451 gram positive and negative bacteria.
452
453 ***4-ASA and 4-AcASA affect cell adhesion and interact with TnaA***
454 The experiments revealed that *E. coli* did not grow in the mineral medium supplemented with
455 4-ASA and 4-AcASA as the sole carbon and energy source, at all tested concentrations
456 (Supplemental table S1). Moreover, MIC assay results indicated that 4-ASA and 4-AcASA
457 concentrations ≤183 μM had no effect on bacterial growth while concentrations ≥1,830 μM
458 inhibited *E. coli* growth (Supplemental figure S1a and S1b). Therefore 4-ASA and 4-AcASA
459 concentrations ≤183 μM were considered sub-inhibitory and used in the cell adhesion assays.
460 At 0.183 μM, 4-ASA and 4-AcASA did not show anti-adhesion activity whereas they significantly
461 affected cell adhesion at 1.83, 18.3 and 183 μM. Indeed, 4-ASA showed the best anti-adhesion
462 performance at 1.83 μM, reducing the number of adhered cells, with respect to the negative control,
463 up to 69.8±4.7 %, while 4-AcASA displayed the best performance at 183 μM, reducing the number
464 of adhered cells, up to 73.5±10.9 % with respect to the negative control (Figure 8a).
465 Aiming to rationalize this experimental evidence, docking calculations of 4-ASA and 4-AcASA in
466 the catalytic site of TnaA were performed. For both ligands, the obtained docking poses reproduced
467 the binding mode hypothesized for SA (Figure 8b). The main differences in the orientation of
468 4-ASA and 4-AcASA were the consequences of the steric clashes between the *para* groups and the
469 enzyme area shaped by Phe464 and Phe39. On the other hand, the enzyme's residues involved in
470 the interaction of SA were similarly engaged by 4-ASA and 4-AcASA. Moreover, the acetyl group
471 of 4-AcASA could create a hydrogen bond with the side chain of Arg103. Performing again
472 docking calculations in the *holo* form of the enzyme, the SA analogues adopted diverse binding

modes, acquiring lower ChemPLP scores than the ones obtained taking into account the closed form of the enzyme.

Discussion

With an approach mimicking nature, SA was considered an attractive compound to be studied as a promising starting point to develop new less-toxic anti-biofilm strategies able to reduce, or even replace, the use of the currently available toxic antimicrobial agents, gradually becoming the treatment choice for the most challenging scenarios. Indeed, this study was addressed to deeply investigate the effect of SA on different key-steps that orchestrate the genesis of virtually every biofilm against the widespread and well-known model bacterium *E. coli*.

According to the biofilm formation process, a promising target for developing innovative anti-biofilm strategies is to avoid the first step of microbial adhesion with a strategy able to repel pioneering cells keeping them in a planktonic form (Villa et al. 2013). The results clearly demonstrate that SA, at concentrations that do not affect the bacterial growth rate, significantly decreases the number of adhered cells up to 68.1±5.6%. According to the anti-biofilm ranges (Cattò et al. 2013), SA displayed moderate anti-adhesion performance at concentrations under 18.3 μM and excellent anti-adhesion performance at 183 μM, in a dose-dependent manner. Previous experiments have shown that *E. coli* capsule polysaccharides can influence bacterial adhesion, and thereby biofilm formation, by shielding of bacterial surface adhesin (Schembri et al. 2004). In the past, SA effects on capsular polysaccharides of bacteria have been observed. Indeed, *E. coli* cultures grown in the presence of SA have shown thinner capsules and lower detectable levels of capsular polysaccharide without significantly inhibiting cell growth (Kam et al. 2009). Accordingly, SA significantly decreased the production of capsular polysaccharides in *Klebsiella pneumoniae* greatly increasing its susceptibility to be phagocytosed by the human immune system (Domenico et al. 1989; Domenico et al. 1992; Salo et al. 1995). In line with these considerations, it is possible that SA affects cell adhesion by a mechanism that involves the production of capsular components.The

second step, which should be considered a promising anti-biofilm target, is the destruction of biofilm integrity by damaging the biofilm matrix (Villa et al. 2013). Indeed, a CDC reactor was employed to generate a lab-scale system and, simulating conditions encountered in-vivo,the biofilm in the presence of 183 μM SA was studied. Experiments revealed a dramatic impact of SA on the biofilm biomass, with a decrease of up to the 97±0.7% in cell numbers compared with the untreated control biofilm. The live/dead assay confirmed that the reduction in the biofilm biomass was achieved by a mechanism that did not affect bacterial viability, suggesting that the anti-biofilm effect was specifically induced by SA. The SA impact on *E. coli* biofilm EPS structure was also studied, focusing attention on the amount of polysaccharides and proteins as these are generally reported to be the major matrix components (Flemming & Wingender 2010). The results showed a significant reduction in the amount of both polysaccharide (49.5±5.5%) and protein (83.9±2.5%) in the SA treated biofilm EPS, compared to the untreated sample. Additional experiments performed by CLSM confirmed the massive impact of SA on biofilm morphology. Indeed, SA treated biofilm resulted significantly decreased in thickness with a mono-layer of dispersed cells and a low amount of polysaccharide matrix. On the contrary, CLSM picture of biofilm grown without SA displayed an intense fluorescence signal corresponding to multi-layers of cells organized in macro-colonies inside a well-structured polysaccharide matrix. In line with our results, Vila and Soto (2012) found that SA decreases the expression of the major outer membrane protein OmpA. OmpA is overexpressed during biofilm maturation and facilitate the transport of polymeric substances required for the formation of the EPS outside the cells. The decrease of the OmpA expression by SA provokes a reduction in the EPS production limiting biofilm development (Vila & Soto 2012). Several studies have reported that *E. coli* produces a biofilm with a highly ordered and complex EPS that provides the three-dimensional structure necessary for the stability of the entire biofilm (Flemming & Wingender 2010; Hung et al. 2013). Polysaccharides were also reported to play a role in *E. coli* adhesion during conversion from planktonic to biofilm mode of growth (Danese et al.

524 2000). Moreover, the role of EPS in *E. coli* pathogenesis has been extensively studied (Sandal
525 2011). Thus it appears clear how much EPS damage by SA could seriously affect biofilm integrity
526 as well as its virulence traits.
527 Finally, another interesting target attempted for novel anti-biofilm compounds is biofilm dispersal
528 by forcing the planktonic state (Villa et al. 2013). Simultaneously with the decrease in biofilm
529 biomass, a significant increase in free-floating cells (78.0±0.6%) was observed in the waste medium
530 after 48 h of SA dynamic treatment. Moreover, the treated biofilm was 3.4±0.6 fold more
531 susceptible to dispersal by external forces, ie PBS, confirming that SA renders biofilm more prone
532 to detachment from the surface. The induction of detachment by SA could greatly enhance the
533 efficacy of antibiotics in the biofilm treatment as, once dispersed from the biofilm, eradicated free-
534 floating bacteria revert to an antibiotic susceptible form (Davey & O'Toole 2000). Given the
535 importance of biofilms in human life, and the increasing antibiotic resistance that, in biofilm, is
536 adaptive and broad spectrum, SA offers considerable potential in the fight against the burgeoning
537 resistance to antibiotics. Indeed, SA might be potentially used to develop more effective therapies
538 based on co-dosed SA and conventional antibiotics (Villa et al. 2013).
539 The overall health of biofilm in terms of level of oxidative stress was also studied in order to
540 explore a possible mechanism of SA action. We found that biofilm treated with SA was more prone
541 to accumulate ROS, in both intracellular (2.3±0.2 fold) and extracellular (2.1±0.3 fold) biofilm
542 compartments, suggesting the presence of an oxidative imbalance between the production of free
543 radicals and the ability of bacteria to counteract their harmful effects through their antioxidant
544 scavenging systems (Gambino & Cappitelli 2016). Accumulation of ROS can result in the
545 peroxidation of lipids, the destruction of cofactors, and the hydroxylation of proteins and nucleic
546 acids, and an excess of them leads to extensive cell damage and eventually cell death, thus seriously
547 affecting biofilm integrity (Čáp et al. 2012). Recently, the role of ROS in some signalling functions
548 involved in biofilm formation has also emerged (Čáp et al. 2012). Indeed, it has been speculated

549 that cells use ROS as a signal or cue to adapt to a changing environment (Gambino & Cappitelli
550 2016). Interestingly, among the *E. coli* proteins targeted by SA, we found that SA interacts with
551 both WrbA and MenI. Indeed, WrbA has been referred to as a flavoprotein, with the enzymatic
552 activity of a NADH:quinone oxidoreductase that prevents the accumulation of ROS maintaining
553 quinones in a fully reduced state, while MenI is involved in the menaquinone biosynthetic pathway
554 (Patridge & Ferry 2006; Latham et al. 2014). Although quinones are essential for normal electron
555 transport, it has been demonstrated that such quinonoids also participate in deleterious redox
556 cycling through direct interactions with single electron acceptors, leading to the accumulation of
557 ROS. However, at present it is not possible to know if ROS accumulation is given by a negative
558 modulation of WrbA and MenI or by other unknown processes. Cattò et al. (2015) demonstrated
559 that the natural compound zosteric acid also interacts with WrbA and proposed a ROS-based
560 mechanism that modulates WrbA activity and results in the decrease of biofilm formation. Thus, the
561 authors do not exclude a similar mechanism in which SA action against biofilm formation is
562 mediated by ROS. Accordingly, some authors have demonstrated that SA plays a direct role in plant
563 defence response through a ROS-dependent pathway in which ROS signals are involved in both
564 upstream and downstream response against pathogen bacteria attacks (Torres et al. 2006, Herrera-
565 Vasquez et al. 2015). Moreover, Rudrappa et al. (2007) reported that catechol, a metabolite of SA,
566 inhibits *Bacillus subtilis* biofilm formation on the *Arabidopsis thaliana* root surface with a ROS-
567 mediated mechanism that further mediates the down regulation of genes involved in biofilm
568 formation.
569 It has been reported that intercellular signalling molecules play a relevant role in biofilm
570 development and detachment. In addition, the ability of ROS to modulate the production of such
571 quorum sensing signals, eg the production of indole, has been shown (Ren et al. 2004). Previous
572 experiments also demonstrated the ability of SA to affect quorum sensing in *B. cereus* and *P.*
573 *fluorescens* biofilm (Lemos et al. 2014). For these reasons, the effects of SA on indole signalling

were investigated. In *E. coli*, indole inhibits biofilm growth, regulates pathogenicity and the expression of multidrug resistance genes (Lee JH & Lee J 2010). Indole also acts as an intercellular signal in many bacterial species, even inducing a response in some species that do not synthesize it (Lee JH & Lee J 2010). Simultaneously with the ROS increase, we found a higher amount of indole in residual *E. coli* biofilm exposed to SA (78.3±16.6%) and that was released into the surrounding environment (7.6±1.4 folds), compared to the untreated sample. Taking into account the docking results, it is possible that SA behaves as a competitive ligand of tryptophan, the natural precursor of the indole biosynthesis pathway (Hu et al. 2010). As indole is an important quorum sensing signal essential for biofilm formation, it is clear that interfering with these communication processes would affect biofilm formation. Intriguingly, among the *E. coli* proteins targeted by SA, the authors found TnaA, the cytoplasmic enzyme that hydrolyses tryptophan to produce indole, together with pyruvate and ammonia, exerting an important role in the biofilm formation process (Hu et al. 2010). Kuczyńska-Wisnik and co-workers (2010) demonstrated that an *E. coli* mutant strain that experiences endogenous oxidative stress showed enhanced expression of tnaA, and increased indole production that delayed biofilm formation. A WrbA role in indole production has also been considered possible (Grandori et al. 1998). Di Martino et al. (2002) found that *E. coli* tnaA mutants show a decreased ability in biofilm formation. Moreover, when they applied the competitive inhibitor (oxindolyl-L-alanine) of tryptophanase to wild-type bacteria they discovered that both indole production and biofilm formation were inhibited in a dose-dependent manner (Di Martino et al. 2003). Molecular modeling studies on TnaA confirm that SA could be a competitive ligand of TnaA, avoiding the binding of tryptophan and its conversion into indole. This is apparently in disaccord with the indole assay results that showed an increased amount of indole within the residual biofilm that is able to tolerate SA, compared to the untreated one. We hypothesize that the overproduction of indole in biofilm grown in the presence of SA might take part in a complex regulatory response system by which bacteria counteract the SA anti-biofilm activity through the

599 TnaA overexpression surviving in the biofilm lifestyle. The increase of indole production in *E. coli*
600 as a stress protection has been always found associated to an increase of the TnaA activity
601 (Kuczynska-Wisnik et al. 2010; Lee JH & Lee J 2010; Villa et al. 2012b). Drug resistance,
602 mediated by the overexpression of the drug molecular target, is recognized as a response pathway
603 for adaptive evolution (Palmer & Kishony 2014). Thus, indole overproduction takes part in a
604 protective mechanism that ensures bacterial survival in the case of stress (Pomposiello et al. 2001;
605 Kuczynska-Wisnik et al. 2010). However, this response was adopted by only 3% of the cells
606 adapted to grow with SA and attached to the coupon. In contrast, on 97% of cells SA displayed its
607 effect against TnaA, providing conditions limiting their adhesion. Since at the concentrations tested
608 SA does not affect bacterial life, the best microbial strategy might be to escape from adverse
609 conditions, rather than activate drug resistance sessile mechanisms, among which is the
610 overexpression of TnaA (Palmer & Kishony 2014).
611 Besides WrbA, MenI and TnaA, the screening of all *E. coli* soluble proteins by a pull-down system
612 combined with a mass spectrometry-based approach revealed that SA in *E. coli* interacts with other
613 proteins. The role of these proteins is not always strictly linked to biofilm formation, but it does
614 involve other systems such as transport, metabolism, motility and the EPS matrix component that
615 might possibly be indirectly connected to the biofilm lifestyle by a complicated network of synergic
616 events.
617 A BLAST search against all non-redundant databases of bacteria revealed that proteins targeted by
618 SA are widespread in a high number of microorganisms. Most returned sequences with 100%
619 identity and an optimal bit score belong to several *E. coli* strains, ranging from harmless gut
620 commensal to intra- or extraintestinal pathogens, which include common colonizers of medical
621 devices and the primary causes of recurrent urogenital infections. Moreover, the BLAST search
622 revealed that all the proteins interactive with SA were highly conserved in different gram positive
623 and gram negative pathogenic bacteria of great concern in the food processing industry (*E. coli*

O157:H7, *Salmonella enterica*, *Shigella sonnei*, *Yersinia enterocolitica*), as well as in medicine and the health-care sector (*K. pneumoniae*, *Vibrio cholerae*), and are responsible for extensive damage to crops in the agricultural field (*Erwinia carotovora*). As proteins targeted by SA in *E. coli* were found to be highly conserved in microorganisms involved in human infections or agricultural diseases, SA could also interact with these proteins, leading to a possible effect on biofilm development. Moreover, as these proteins are highly conserved in multiple pathogens, SA could be potentially used with a broad-spectrum activity against mixed infections. In this direction, salicylates have been shown to be of benefit in some biofilm-associated diseases including *P. aeruginosa*, *K. pneumoniae* and *S. epidermidis* (Faber & Wolff 1993; Teichberg et al. 1993; Muller et al. 1998; Prithiviraj et al. 2005; El-Banna et al. 2012).

Even though additional evidence is needed to confirm a functional interaction between the pulled-down proteins and SA, to the best of our knowledge this is the only study investigating the putative molecular targets implicated in SA anti-biofilm responses. It is very probable that the discovery of bacterial targets for SA could greatly advance the understanding of SA's important signalling pathway in plants, as well as enlarge on the present perspective for its widespread use as a powerful anti-biofilm agent (Kumar 2014).

Given the important results obtained with SA, two SA derivatives, ie 4-ASA and 4-AcASA, have been preliminary tested for their anti-biofilm performances. Indeed, 4-ASA has been successfully used for over a century in the treatment of multidrug resistant tuberculosis and it is highly effective against ulcerative colitis (Dhaneshwar 2014). Experiments revealed that 4-ASA and 4-AcASA significantly decrease the number of adhered cells up to 69.8±4.7 % and 73.5±10.9 % respectively at a concentration that do not affect bacterial growth rate, showing performances in line with those achieved by SA. However, both the molecules started to display their anti-adhesion properties one order of magnitude more concentrated (1.83 μM) in comparison to SA. Consistently with the hypothesis that 4-ASA and 4-AcASA could display a mechanism of action similar to SA, as they

649 share the same chemical backbone, docking calculations suggested that the presence of the *p*-amino
650 groups only slightly influenced the binding mode predicted for such compounds. In fact, 4-ASA
651 and 4-AcASA involve essentially the same TnaA residues previously expected for SA.
652
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655
656 **References**
657 Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of
658 protein utilizing the principle of protein-dye binding. Anal Biochem. 72:248−254.
659 Čáp M, Vachova L, Palkova Z. 2012. Reactive oxygen species in the signaling and adaptation of
660 multicellular microbial communities. Oxid Med Cell Longev. 2012:976753.
661 Cappitelli F, Polo A, Villa F. 2014. Biofilm formation in food processing environments is still
662 poorly understood and controlled. Food Eng Rev. 6:29−42.
663 Case DA, Darden TA, Cheatham III TE., Simmerling CL, Wang J, Duke RE, LuoR, Walker RC,
664 Zhang W, Merz KN, et al. 2012. Amber 12. San Francisco (CA): University of California.
665 Cattò C, Dell'Orto S, Villa F, Villa S, Gelain A, Vitali A, Marzano V, Baroni S, Forlani F,
666 Cappitelli F. 2015. Unravelling the structural and molecular basis responsible for the anti-biofilm
667 activity of zosteric acid. PLoS One. 10:e0131519.
668 Chow S, Gu K, Jiang L, Nassour A. 2011. Salicylic acid affects swimming, twitching and swarming
669 motility in *Pseudomonas aeruginosa*, resulting in decreased biofilm formation. JEMI. 15:25−29.
670 Damman CJ. 2013. Salicylates and the microbiota: a new mechanistic understanding of an ancient
671 drug's role in dermatological and gastrointestinal disease. Drug Develop Res. 74:344−352.
672 Danese PN, Pratt LA, Kolter R. 2000. Exopolysaccharide production is required for development of
673 *Escherichia coli* K-12 biofilm architecture. J Bacteriol. 182:3593−3596.

674 Davey ME, O'Toole G A. 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol
675 Mol Biol Rev. 64:847−867.Dhaneshwar SS. 2014. Colon-specific prodrugs of 4-aminosalicylic acid
676 for inflammatory bowel disease. World J Gastroenterol. 20:3564−3571
677 Di Martino P, Merieau A, Phillips R, Orange N, Hulen C. 2002. Isolation of an *Escherichia coli*
678 strain mutant unable to form biofilm on polystyrene and to adhere to human pneumocyte cells:
679 involvement of tryptophanase. Can J Microbiol. 48:132−137.
680 Di Martino P, Fursy R, Bret L, Sundararaju B, Phillips RS. 2003. Indole can act as an extracellular
681 signal to regulate biofilm formation of *Escherichia coli* and other indole-producing bacteria. Can J
682 Microbiol. 49:443−449.
683 Di Pasqua R, Mamone G, Ferranti P, Ercolini D, Mauriello G. 2010. Changes in the proteome of
684 *Salmonella enterica* serovar Thompson as stress adaptation to sublethal concentrations of thymol.
685 Proteomics. 10:1040−1049.
686 Domenico P, Schwartz S, Cunha BA. 1989. Reduction of capsular polysaccharide production in
687 *Klebsiella pneumoniae* by sodium salicylate. Infect Immun. 57:3778−82.Domenico P, Salo RJ,
688 Straus DC, Hutson JC, Cunha BA. 1992. Salicylate or bismuth salts enhance opsonophagocytosis of
689 *Klebsiella pneumoniae*. Infection. 20:66−72.
690 El-Banna T, Sonbol FI, Abd El-Aziz AA, Abo-Kamar A, Seif-Eldin DW. 2012. Effect of the
691 combination of salicylate with aminoglycosides on bacterial adhesion to urinary catheters. Int Res J
692 Pharm. 2:39−45.
693 Farber BF, Wolff AG. 1993. Salicylic acid prevents the adherence of bacteria and yeast to silastic
694 catheters. J Biomed Mater Res. 27:599−602.
695 Frisch M J, Trucks GW, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JR, Scalmani G, Barone
696 V, Mennucci B, Petersson GA, et al. 2009. Gaussian 09. Revision A.02. Wallingford (CT):
697 Gaussian, Inc.
698 Flemming HC, Wingender J. 2010. The biofilm matrix. Nat Rev Microbiol. 8:623−633.

699 Hartshorn MJ, Verdonk ML, Chessari G, Brewerton SC, Mooij WT, Mortenson PN, Murray CW.
700 2007. Diverse, high-quality test set for the validation of protein-ligand docking performance. J Med
701 Chem. 50:726−741.
702 Gambino M, Cappitelli F. 2016. Mini-review: Biofilm responses to oxidative stress. Biofouling.
703 32:167−178.
704 Grandori R, Khalifah P, Boice JA, Fairman R, Giovanielli K, Carey J. 1998. Biochemical
705 characterization of WrbA, founding member of a new family of multimeric flavodoxin-like
706 proteins. J Biol Chem. 273:20960−20966.
707 Herrera-Vasquez A, Salinas P, Holuigue L. 2015. Salicylic acid and reactive oxygen species
708 interplay in the transcriptional control of defense genes expression. Front Plant Sci. 6:9.
709 Hu M, Zhang C, Mu Y, Shen Q, Feng Y. 2010. Indole affects biofilm formation in bacteria. Indian J
710 Microbiol. 50:362−368.
711 Hung C, Zhou Y, Pinkner JS, Dodson KW, Crowley JR, Heuser J, Chapman MR, Hadjifrangiskou
712 M, Henderson JP, Hultgren SJ. 2013. *Escherichia coli* biofilms have an organized and complex
713 extracellular matrix structure. MBio. 4:e00645−00613.
714 Jakubowski W, Bilinski T, Bartosz G. 2000. Oxidative stress during aging of stationary cultures of
715 the yeast *Saccharomyces cerevisiae*. Free Radic Biol Med. 28:659−664.
716 Kam J, Luo XL, Song HA. 2009. Effects of reduced capsular polysaccharide on kanamycin
717 resistance in *Escherichia coli* b23 cells. JEMI. 13:22−28.
718 Kogan A, Raznov L, Gdalevsky GY, Cohen-Luria R, Almog O, Parola AH, Goldgur Y. 2015.
719 Structures of *Escherichia coli* tryptophanase in holo and 'semi-holo' forms. Acta Crystallogr F
720 Struct Biol Commun. 71:286−290.
721 Ku SY, Yip P, Howell PL. 2006. Structure of *Escherichia coli* tryptophanase. Acta Crystallogr D
722 Biol Crystallogr. 62:814−823.
723 Kuczynska-Wisnik D, Matuszewska E, Laskowska E. 2010. *Escherichia coli* heat-shock proteins

724 IbpA and IbpB affect biofilm formation by influencing the level of extracellular indole.
725 Microbiology. 156:148−157.
726 Kumar D. 2014. Salicylic acid signaling in disease resistance. Plant Sci. 228:127−134.
727 Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of
728 bacteriophage T4. Nature. 227:680−685.
729 Lagonenko L, Lagonenko A, Evtushenkov A. 2013. Impact of salicylic acid on biofilm formation
730 by plant pathogenic bacteria. J Biol Earth Sci. 3:B176−B181.
731 Latham JA, Chen D, Allen KN, Dunaway-Mariano D. 2014. Divergence of substrate specificity and
732 function in the *Escherichia coli* hotdog-fold thioesterase paralogs YdiI and YbdB. Biochemistry.
733 53:4775−4787.
734 Lee JH, Lee J. 2010. Indole as an intercellular signal in microbial communities. FEMS Microbiol
735 Rev. 34:426−444.
736 Lemos M, Borges A, Teodósio J, Araújo P, Mergulhão F, Melo L, Simões M. 2014. The effects of
737 ferulic and salicylic acids on *Bacillus cereus* and *Pseudomonas fluorescens* single- and dual-species
738 biofilms. Int Biodeterior Biodegradation. 86:42−51.
739 Masuko T, Minami A, Iwasaki N, Majima T, Nishimura SI, Lee YC. 2005. Carbohydrate analysis
740 by a phenol-sulfuric acid method in microplate format. Anal Biochem. 339:69−72.
741 Merrit JH, Kadouri DE, O'Toole GA. 2005. Growing and analyzing static biofilms. Curr Protoc Microbiol.
742 01:Unit–1B.1.
743 Muller E, Al-Attar J, Wolff AG, Farber BF. 1998. Mechanism of salicylate-mediated inhibition of
744 biofilm in *Staphylococcus epidermidis*. J Infect Dis. 177:501−503.
745 Palmer AC, Kishony R. 2014. Opposing effects of target overexpression reveal drug mechanisms.
746 Nat Commun. 5:4296.
747 Patridge EV, Ferry JG. 2006. WrbA from *Escherichia coli* and *Archaeoglobus fulgidus* is an
748 NAD(P)H:quinone oxidoreductase. J Bacteriol. 188:3498−3506.

749 Pearson WR. 2013. An introduction to sequence similarity ("homology") searching. Curr Protoc
750 Bioinformatics. Chapter 3:Unit3.1.
751 Pomposiello PJ, Bennik MHJ, Demple B. 2001. Genome-wide transcriptional profiling of the
752 Escherichia coli responses to superoxide stress and sodium salicylate. J Bacteriol. 183:3890−3902.
753 Polo A, Foladori P, Ponti B, Bettinetti R, Gambino M, Villa F, Cappitelli F. 2014. Evaluation of
754 zosteric acid for mitigating biofilm formation of *Pseudomonas putida* isolated from a membrane
755 bioreactor system. Int J Mol Sci. 15:9497−9518.
756 Prithiviraj B, Bais HP, Weir T, Suresh B, Najarro EH, Dayakar BV, Schweizer HP, Vivanco JM.
757 2005. Down regulation of virulence factors of *Pseudomonas aeruginosa* by salicylic acid attenuates
758 its virulence on *Arabidopsis thaliana* and *Caenorhabditis elegans*. Infect Immun. 73:5319−5328.
759 Raskin I. 1992. Role of salicylic-acid in plants. Annu Rev Plant Physiol Plant Mol Biol.
760 43:439−463.
761 Ren D, Bedzyk LA, Thomas SM, Ye RW, Wood TK. 2004. Gene expression in *Escherichia coli*
762 biofilms. Appl Microbiol Biotechnol. 64:515−524.
763 Rosenberg LE, Carbone AL, Römling U, Uhrich KE, Chikindas ML. 2008. Salicylic acid-based
764 poly(anhydride esters) for control of biofilm formation in *Salmonella enterica* serovar
765 Typhimurium. Lett Appl Microbiol. 46:593–599.
766 Rudrappa T, Quinn WJ, Stanley-Wall NR, Bais HP. 2007. A degradation product of the salicylic
767 acid pathway triggers oxidative stress resulting in down-regulation of *Bacillus subtilis* biofilm
768 formation on *Arabidopsis thaliana* roots. Planta. 226:283−297.
769 Salo RJ, Domenico P, Tomás JM, Straus DC, Merino S, Benedí VJ, Cunha BA. 1995. Salicylate-
770 enhanced exposure of *Klebsiella pneumoniae* subcapsular components. Infection. 23:371−377.
771 Sandal I, Inzana TJ, Molinaro A, De Castro C, Shao JQ, Apicella MA, Cox AD, St Michael F, Berg
772 G. 2011. Identification, structure, and characterization of an exopolysaccharide produced by
773 *Histophilus somni* during biofilm formation. BMC Microbiol. 11:186.

774 Schembri MA, Dalsgaard D, Klemm P. 2004. Capsule shields the function of short bacterial
775 adhesins. J Bacteriol. 186:1249−57.
776 Teichberg S, Farber BF, Wolff AG, Roberts B. 1993. Salicylic acid decreases extracellular biofilm
777 production by *Staphylococcus epidermidis*: electron microscopic analysis. J Infect Dis.
778 167:1501−1503.
779 Torres MA, Jones JDG, Dangl JL. 2006. Reactive oxygen species signaling in response to
780 pathogens. Plant Physiol. 141:373−378.
781 Trott O, Olson AJ. 2010. AutoDock Vina: improving the speed and accuracy of docking with a new
782 scoring function, efficient optimization, and multithreading. J Comput Chem. 31:455−461.
783 Vila J, Soto SM. 2012. Salicylate increases the expression of marA and reduces in vitro biofilm
784 formation in uropathogenic *Escherichia coli* by decreasing type 1 fimbriae expression. Virulence
785 3:280−285.
786 Villa F, Albanese D, Giussani B, Stewart PS, Daffonchio D, Cappitelli F. 2010. Hindering biofilm
787 formation with zosteric acid. Biofouling. 26:739−752.
788 Villa F, Remelli W, Forlani F, Gambino M, Landini P, Cappitelli F. 2012a. Effects of chronic sub-
789 lethal oxidative stress on biofilm formation by *Azotobacter vinelandii*. Biofouling. 28:823−833.
790 Villa F, Remelli W, Forlani F, Vitali A, Cappitelli F. 2012b. Altered expression level of
791 *Escherichia coli* proteins in response to treatment with the antifouling agent zosteric acid sodium
792 salt. Environ Microbiol. 14:1753−1761.
793 Villa F, Villa S, Gelain A, Cappitelli F. 2013. Sub-lethal activity of small molecules from natural
794 sources and their synthetic derivatives against biofilm forming nosocomial pathogens. Curr Top
795 Med Chem. 13:3184−3204.
796 Vlot AC, Dempsey DA, Klessig DF. 2009. Salicylic acid, a multifaceted hormone to combat
797 disease. Annu Rev Phytopathol. 47:177−206.
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Tables

Table 1. Planktonic growth in the presence of SA as the sole carbon and energy source. Data represent the mean ± standard deviation of three independent measurements. Different superscript letters indicate statistically significant differences (Tukey's HSD, $p \leq 0.001$) between the means of three independent replicates. The positive controls were set up with the mineral medium supplemented with glucose at both 1,830 μM and 3,000 μM.

Concentration (μM)	**A_{600} ml^{-1}**
0	0.031 ± 0.003^{a}
0.183	0.030 ± 0.006^{a}
1.83	0.026 ± 0.006^{a}
18.3	0.029 ± 0.005^{a}
183	0.028 ± 0.006^{a}
1,830	0.028 ± 0.006^{a}
1,830 (glucose)	0.121 ± 0.007^{b}
3,000 (glucose)	0.172 ± 0.011^{c}

Table 2. Adhered cells without and with SA at sub-lethal concentration. Data represent the mean ± standard deviation of the number of adhered cells cm^{-2}. Different superscript letters indicate significant differences (Tukey's HSD, $p \leq 0.001$) between the means of three independent replicates. Percentage reduction with respect to the negative control is calculated as (SA data – negative control data) x 100 / negative control data.

Concentration (µM)	**No. adhered cells $cm^{-2} \times 10^7$**	**Reduction %**
0	5.71 ± 0.59^{a}	0.0 ± 0.0^{a}
0.183	4.23 ± 0.55^{b}	25.9 ± 3.4^{b}
1.83	4.02 ± 0.85^{b}	29.6 ± 6.4^{b}
18.3	3.59 ± 0.64^{b}	37.1 ± 6.6^{b}
183	1.81 ± 0.32^{c}	68.1 ± 5.6^{c}

Table 3. Mass spectrometry protein identification. a: P, primary hit: proteins found with the best score in each band slice. S, secondary hit: proteins never present with the best score in any band slice. b: Accession number of the UniProtKB/Swiss-Prot database. c: Protein identification's SEQUEST HT score. d: Percentage of protein sequence covered by the identified peptides. Identified proteins are ranked according to the molecular weight inside each hit category.

Hit position (a)	Accession (b)	Protein name/gene name/synonyms gene name	Score (c)	Coverage % (d)
P	P0A6F5	60 kDa chaperonin/*gro*L/*groEL*, *mopA*	1246.82	87.04
	P0A9C5	Glutamine synthetase/*gln*A	278.58	65.67
	P0A853	Tryptophanase/*tna*A	256.19	58.39
	P07913	L-threonine 3-dehydrogenase/*tdh*	513.15	59.53
	P45523	FKBP-type peptidyl-prolyl cis-trans isomerase/*fkp*A/*yzzS*	153.73	37.78
	P0A8G6	NAD(P)H dehydrogenase (quinone)/*wrb*A	988.33	71.72
	P0A998	Ferritin-1/*ftn*A/*ftn*, *gen-165*, *rsgA*	410.08	71.52
	P0A6Q6	3-hydroxyacyl-[acyl-carrier-protein] dehydratase/*fabZ*/*sefA*, *yaeA*	262.49	50.33
S	P67910	ADP-L-glycero-D-manno-heptose-6-epimerase/*hld*D/*htrM*, *rfaD*, *waaD*	52.00	35.16
	P0A9L3	FKBP-type 22 kDa peptidyl-prolyl cis-trans isomerase/*fkl*B/ ytfC	50.30	48.54
	P33012	DNA gyrase inhibitor/*sbm*C/*gyrI, yeeB*	118.37	33.76
	P0AG86	Protein-export protein/*sec*B	87.53	50.97
	P77781	1,4-dihydroxy-2-naphthoyl-CoA hydrolase/*men*I/*ydi*I	68.02	47.06

Figures

Figure 1. *E. coli* planktonic growth without and with SA. a: A_{600}-based growth curve of *E. coli* without and with SA at different concentrations. b: Growth parameters lag time length (λ), maximum specific growth rate (μ_m) and the Goodness of Fit (R^2) obtained by the Gompertz model. Data represent the mean ± standard deviation of three independent measurements. Different superscript letters indicate statistically significant differences (Tukey's HSD, $p \leq 0.001$) between the means of three independent biological replicates.

Figure 2. Biofilm structural development without and with 183 µM SA. a: Viable adhered cells obtained by plate count viability assay. b: Relative viability within the biofilm. c: Proteins and polysaccharides in the matrix. Data in a, b and c represent the mean ± standard deviation of three independent biological replicates; the histogram provides the *p*-values obtained by Student's t-test analysis; a star (*) indicates statistically significant difference at the 99.9% confidence level between control and treated samples. d, e: Representative side views of 3D reconstructed CLSM images of biofilm grown without (d) and with SA (e) (λ_{ex} at 488 nm, and λ_{em}<530 nm, 60×, 1.0 NA water immersion objective). Live cells were stained green with Syber green I, whereas the polysaccharide matrix was stained red with Texas Red-labelled ConA. Scale bar=40 µm.

Figure 3. Biofilm dispersion without and with 183 µM SA. a: A_{600} of the waste medium released by the CDC after 48 h of dynamic phase. b: Surface dispersion of pre-grown biofilm soaked in PBS for 24 h. Data represent the mean ± standard deviation of three independent biological replicates. The histograms provide the *p*-values obtained by Student's t-test analysis. A star (*) indicates statistically significant difference at the 99.9% confidence level between control and treated samples.

Figure 4. Level of ROS without and with 183 µM SA. Data represent the mean ± standard deviation of three independent biological replicates. The histogram provides the *p*-values obtained

879 by Student's t-test analysis. A star (*) indicates statistically significant difference at the 99.9%
880 confidence level between control and treated samples. A.U.: arbitrary unit.
881 **Figure 5. Indole signalling.** a: Extracellular, intracellular and in the output waste medium indole in
882 the presence of 183 μM SA. Data represent the mean ± standard deviation of three independent
883 biological replicates. The histograms provide the *p*-values obtained by Student's t-test analysis. A
884 star (*) indicates statistically significant difference at the 99.9% confidence level between control
885 and treated samples. b: Connolly accessible surface area of one monomer of TnaA. The partial
886 charges of the outward residues are projected: positively and negatively charged areas are colored
887 blue and red, respectively. The arrow shows the location of the substrate and the PLP binding sites.
888 c: Binding mode of SA (cyan sticks) in the catalytic site of tryptophanase. d: Binding mode of one
889 sulphate ion found in the 2C44 X-ray structure (yellow and red stick models). Yellow dotted lines
890 in panels b and c represent the hydrogen bonds.
891 **Figure 6.** a: Base peak chromatogram of mass spectrometry analysis of hydrolysed 4-ASA/matrix;
892 *m/z* value on main peak and spectra registered between 8.96 and 9.27 min are evidenced. The
893 154.049 *m/z* ion corresponds (ΔM = 0.001 Da) to the theoretical *m/z* value of the [M+H]+ of 4-
894 ASA; 83.060 and 214.088 ions are acetonitrile (solvent) and n-BBS, n-butyl benzenesulfonamide,
895 (plasticizer) signals, respectively. NL, normalization level. b: Coomassie Blue stained SDS-PAGE
896 of main fractions from a representative protein-pull-down experiment. CE, crude extract (35 μg); U,
897 unbound fraction (35 μg); W1, W2, washing step fractions; E1-E4, elution step fractions. W and E
898 samples were TCA-precipitated from 870 μl fraction aliquots. The Mrs and positions of marker
899 proteins are reported. Bands submitted to MS analysis are indicated by asterisks.
900 **Figure 7.** Protein identity from NCBI-BLASTP search against all non-redundant databases of
901 Bacteria using each one of the protein targeted by SA in *E. coli* as a query. Returned sequences
902 showing an identity <30% and an expect-value threshold $>10^{-5}$ were considered not significantly
903 similar to the query sentence (n. s.). Returned sequences with a bit score ≥200 were considered with

an optimal level of similarity with the query sentence (red), between an 80 and 200 with good level of similarity (pink), between 50 and 80 with a moderate level of similarity (green) and <50 with a low level of similarity (blu). +: gram positive strain; -: gram negative strain.

Figure 8. Cell adhesion and TnaA interaction of 4-ASA and 4-AcASA. a: Adhered cells cm^{-2} without and with 4-ASA and 4-AcASA at sub-lethal concentrations. Data represent the mean ± standard deviation of three independent measurements. Different superscript letters indicate statistically significant differences (Tukey's HSD, $p \leq 0.001$) between the means of three independent replicates. b: Supposed binding mode of 4-ASA (yellow sticks) and 4-AcASA (magenta sticks) in the catalytic site of TnaA in the closed conformation. SA is showed as thin cyan sticks whereas yellow dotted lines represent the hydrogen bonds between the ligands and the enzyme (green sticks).