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Short communication

A method to map changes in bacterial surface composition induced by regulatory RNAs in *Escherichia coli* and *Staphylococcus aureus*

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ABSTRACT

We have adapted a method to map cell surface proteins and to monitor the effect of specific regulatory RNAs on the surface composition of the bacteria. This method involves direct labeling of surface proteins of living bacteria using fluorescent dyes and a subsequent separation of the crude extract by 2D gel electrophoresis. The strategy yields a substantial enrichment in surface proteins over cytoplasmic proteins. We validated this method by monitoring the effect of the regulatory RNA MicA in *Escherichia coli*, which regulates the synthesis of several outer membrane proteins, and highlighted the role of *Staphylococcus aureus* RNAIII for the maintenance of cell wall integrity.

1. Introduction

Bacteria interact constantly with their environment via surface-associated proteins to fulfill a range of functions including adhesion, environmental sensing, and nutrient transport. A major drawback in studies of surface proteins is their difficulty of extraction. Different tools have been employed to enrich membrane fractions, but none are able to give a full map of membrane proteins, due to difficulty of experimental conditions settings, contamination or significant loss of these proteins through fractionation [1–3].

Here we have adapted a method that allows specific labeling of exposed lysine residues of surface proteins from living bacteria followed by 2D gel electrophoresis [4,5]. This method was applied to *Escherichia coli* and *Staphylococcus aureus* to follow the dynamics of cell surface composition that could be controlled by the expression of regulatory RNAs. We have analyzed two RNAs that are known to modify the surface properties of the bacteria in order to respond to environmental conditions or to escape the host-defense mechanisms. In *E. coli*, the sigma E-induced MicA senses envelope stress and represses the synthesis of several outer membrane proteins (OmpA, OmpX, LamB, OmpW, Tsx), and of the response regulatory protein PhoP [6–8], while in *S. aureus*, the multiple-target regulatory RNAIII controls the switch between the expression of surface proteins and excreted toxins [9,10]. Both RNAs regulate gene expression at the post-transcriptional level by binding in the 5′ region of mRNA targets. The results reported show that the method is suitable to gain insights into cell envelope composition and its dynamics upon a variety of experimental conditions.

2. Methods

The *E. coli* strain MC4100 was transformed with a plasmid derived from PZE12-luc, either lacking insert (as control) or expressing wild-type MicA (Table S1), and was grown at 37 °C in LB medium supplemented with ampicillin until late exponential phase (OD = 1). *S. aureus* RN6390 strain, derived from NCTC8325, and the isogenic LUG950 strain (deletion of *rna*III gene, Δ*rna*III, see

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Table S1) were grown in BHI medium until late exponential phase (OD = 5).

Cells from 10 ml (*E. coli*) or 2.5 ml of culture (*S. aureus*) were centrifuged at 4500 g at 4 °C, washed twice with 1 ml of PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 8) and resuspended in 200 µl of the PBS buffer. The Cy Dyes 2, 3 and 5 were prepared freshly in anhydrous dimethylformamide. Accessible lysines of cell surface proteins were labeled with 200 pmol of Cy Dye for 30 min at 4 °C in the dark, and the reaction was stopped by the addition of 20 µl of 10 mM lysine for 10 min at 4 °C. Cells were pelleted by centrifugation at 12,000 g for 1 min.

E. coli cells were disrupted by the addition of 1 ml of Trizol for 5 min at 20 °C. 100 µl of 1-bromo 3-chloropropane chloroform was added to the samples, incubated for 5 min at 20 °C and centrifuged for 10 min at 12,000 g. Cold ethanol (200 µl) was added to the pink phase and the samples were incubated for 5 min at 20 °C, and centrifuged for 5 min at 2000 g. Proteins from the supernatant were precipitated with 7 volumes of cold acetone for at least 2 h at −20 °C. Disruption of *S. aureus* cells was achieved by 30 min incubation at 37 °C in 200 µl of lysis buffer (10 mM Tris−HCl pH 7.5, 20 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 50 µg/ml lysostaphin, protease inhibitors cocktail (Roche), 2 U of DNase, and 2 U of RNase). Proteins were dissolved in the 2D buffer (7 M urea, 2 M thiourea, 50 mM DTT, 4% CHAPS) in a total volume of 400 µl. Protein concentration was measured using the Bio-Rad Bradford assay and 300 µg of total protein extract was used for 2D gel fractionation.

2D gel analyses were carried out according to the manufacturer's recommendations (Biorad). After electrophoresis, gels were scanned on a DIGE imager (GE Healthcare) and stained with colloidal blue to visualize the whole set of proteins. Statistical analysis of protein variation was performed for at least two independent replicates. After scanning, the data were analyzed with PDQuest software (Biorad). Protein spots were excised from 2D-gels, trypsin digested, and MALDI mass spectroscopy was carried out on an Autoflex III (Bruker-Daltonik GmbH, Germany) matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF) used in reflector positive mode. Experimental details are given in the Supplementary Information.

3. Results and discussion

3.1. The three Cy-Dyes differentially label surface proteins on living cells

Two previous studies in human [4] and *Legionella pneumophila* [5] have shown that the three Cy Dyes specifically label membrane proteins. However, using two complementary approaches we have verified that the three dyes did not efficiently penetrate into the Gram-negative *E. coli* and the Gram-positive *S. aureus* cells (Supplementary Information). Cy Dye labeled bacteria submitted to imaging with a Zeiss LSM780 laser scanning confocal microscope reproducibly showed that the fluorescence accumulated predominantly at the periphery of the bacteria (Figure S1A). Consistently, the Cy Dye labeling was more efficient on membrane-enriched protein fractions than on cytoplasmic protein fractions (Figure S1B, see Supplementary Information). All in all, these data strongly suggested that the Cy Dyes penetrate only poorly in *E. coli* or *S. aureus* live cells.

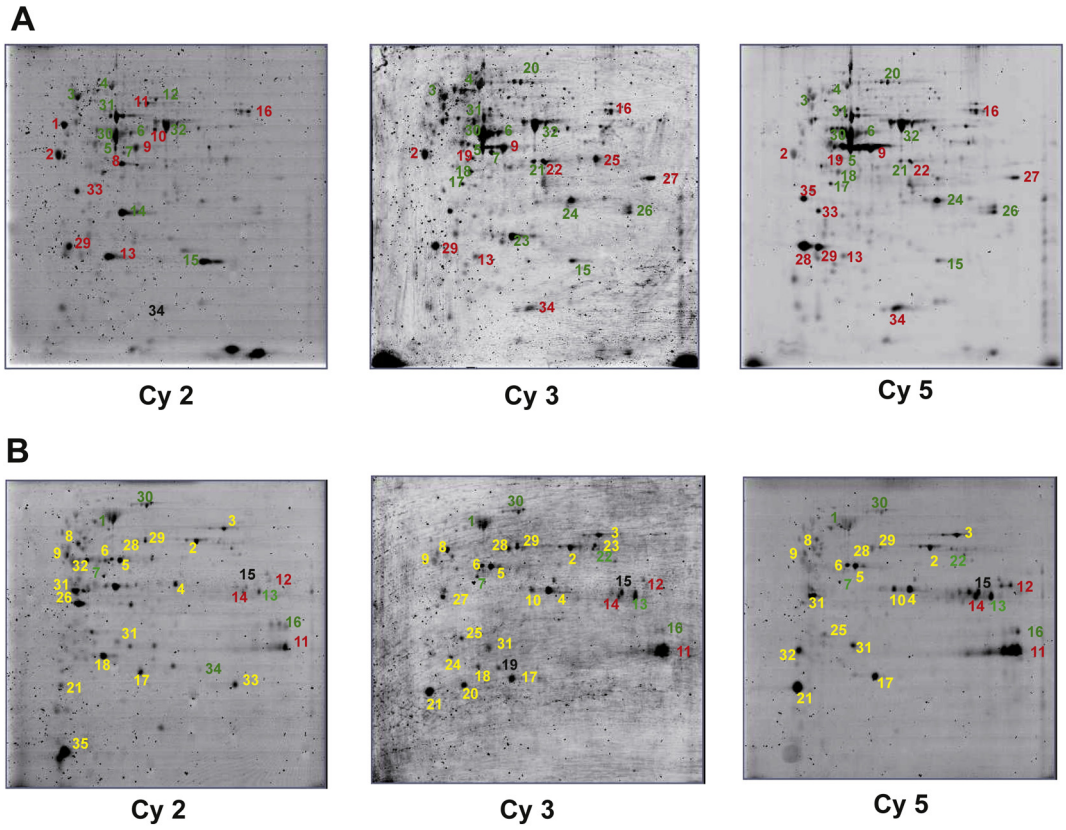
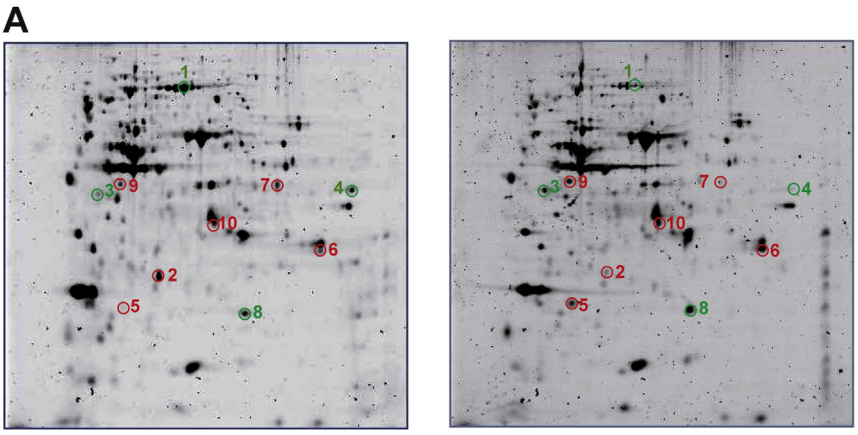


Fig. 1. Labeling of the surface proteins in living *Escherichia coli* and *Staphylococcus aureus* cells. (A, B) Two-dimensional gel fractionation of Cy Dye labeled proteins from *E. coli* (A) and *S. aureus* (B) were performed. Labeling was done with cyanine 2, 3 and 5 (Cy 2, Cy 3 and Cy 5, respectively). All spots corresponding to membrane proteins and cell surface proteins are numbered in red while the spots corresponding to cytoplasmic proteins are in green. Spots numbered in yellow color corresponded to *S. aureus* proteins found in different compartments of the cells as demonstrated from a quantitative proteomic analysis [1]. The program Psort (psort.hgc.jp) was used to predict the localization of *S. aureus* proteins. For more details, the identity of the proteins and their localization are given in Tables S2 and S3.

A comparative analysis of the target proteins labeled by the three fluorophores was then performed. Live *E. coli* and *S. aureus* cells were treated separately with each of the three Cy Dyes. After labeling, the cells were collected by low-speed centrifugation to remove excess of unbound dye. Analysis of the supernatant showed that it contained less than 3% of the total proteins from the samples, thus confirming that the labeling protocol did not cause extensive lysis of the cells. The samples were submitted to 2D gel electrophoresis and subsequently scanned to visualize the fluorescent proteins, which were then identified by MALDI-TOF (Fig. 1). For *E. coli*, the data showed a significant enrichment in membrane proteins over cytoplasmic proteins (Figure S1, Table S2). For *S. aureus*, among the labeled proteins, only 20% of them were predicted to be located in the membrane (Table S3). A quantitative proteome of *S. aureus* COL strain [1] revealed that many proteins with a cytoplasmic location were also found temporary or permanently associated with the membrane sub-fractions (Fig. 1, Table S3). Because the Cy Dyes penetrate poorly in *S. aureus* (Figure S1), it might be possible that these proteins are transiently located at the membrane, as it is the case for several enzymes involved in the respiration (Table S3). Rather unexpectedly, we found that the three Cy Dyes displayed differential labeling properties (Fig. 1) although we did not find specific signatures for each Cy Dye (outer/inner membrane proteins). These variations most likely reflect subtle differences in their structures because the three Cy Dyes gave comparable labeling efficiencies when proteins from



Spot number	Protein name	Gene name	fold change	Localisation
1	pyruvate formate lyase I	*pflB*	11	cytoplasm
2	*NAD(P)H:quinone oxidoreductase*	*wrba*	10	membrane
3	Transaldolase B	*talB*	0,15	cytoplasm
4	hypothetical protein Z2603	*ydgH*	7	cytoplasm
5	*outer membrane protein X*	*ompX*	0,2	membrane
6	*ABC-type organic solvent transporter*	*yrbC*	0,2	membrane
7	glyceraldehyde-3-phosphate dehydrogenase	*gapA*	3	membrane
8	50S ribosomal protein L9	*rplI*	0,43	cytoplasm
9	*regulator of ftsI, penicillin binding protein 3, septation function*	*mreB*	0,5	membrane
10	D-Ribose-Binding Protein	*rbsB*	0,5	membrane

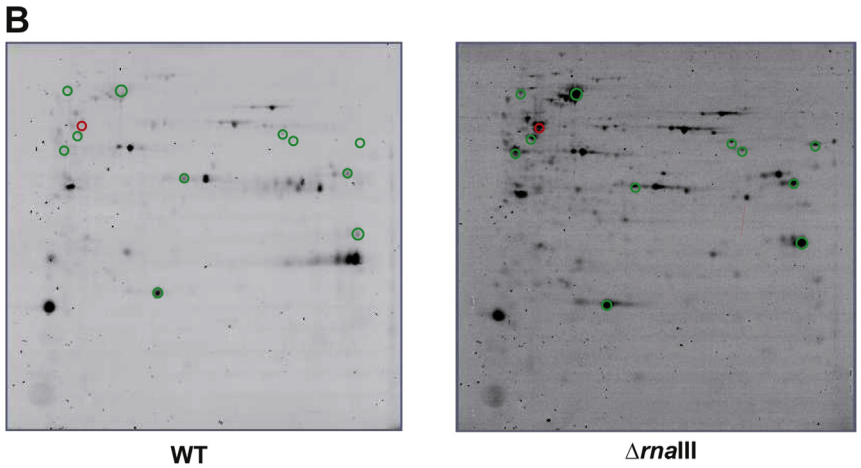


Fig. 2. Effect of regulatory RNA expression on the surface protein patterns in *E. coli* and *S. aureus*. (A) Two-dimensional gel electrophoresis of Cy Dye 5 labeled proteins from the *E. coli* wild-type strain expressing high levels of MicA (+MicA; overproduction of MicA encoded on a plasmid) and from the same strain expressing low levels of MicA (-MicA; chromosomal level of MicA expression). The difference in MicA expression is shown in Figure S2. Cytoplasmic proteins are circled in green and membrane proteins are in red. The names of the proteins and their corresponding genes are given in the table. The quantification (fold change) represents the ratio of protein yield obtained from the wild-type strain expressing high levels of MicA from a plasmid over that of wild-type strain. The data were reproducibly found in three independent experiments. We have only considered the effects above 2-fold. (B) Two-dimensional gel electrophoresis of Cy 5 labeled proteins from *S. aureus* wild-type (WT) strain expressing high levels of RNAIII and the isogenic LUG950 strain deleted of the *rna*III gene (Δ*rna*III). The green spots correspond to cytoplasmic proteins that are predominantly observed in the Δ*rna*III mutant strain, and only protein A is shown in red. Other details are given in Figure S3.

crude extracts were treated under denaturing conditions, typically in buffers containing urea or detergent (results not shown). Therefore, it is of importance to perform comparative proteomics on living cells with the same Cy Dye. Although the approach is sensitive, several outer membrane proteins such as OmpA in *E. coli* were not detected (Table S2). The most likely explanation is the inaccessibility of lysine residues of OmpA to the dyes. Indeed, the crystal structure of OmpA has revealed that only one lysine could be exposed at the surface of the cell [11].

3.2. The Cy dye labeling method can map changes of the cell surface

In the following experiments, Cy 5 was used for differential proteomics analysis, because we observed that slightly more membrane proteins were labeled with this dye (Fig. 1; Tables S2 and S3). The cell surface proteomes were analyzed for wild-type *E. coli* cells expressing low levels of MicA and for the same strain expressing high levels of MicA from a multicopy plasmid. Ten proteins with altered yields were reproducibly observed (Fig. 2(A)). Six of them are known to be anchored to membranes (YdgH, OmpX, YrbC/MlaC, RbsB, GapA, and WrbA) or associated with membrane proteins (MreB), while the others (PflB, and TalB) are presumed to be cytoplasmic. Because MicA regulates translation and degradation of mRNA targets by binding to their 5′ untranslated regions, we analyzed by Northern blot experiments the levels of several mRNAs (*omp*X, *yrb*C/*mla*C, *mre*B, and *wrb*A) that were activated (*wrb*A) or repressed (*omp*X, *yrb*C/*mla*C, *mreB*) by MicA at the post-transcriptional level (Figure S2A). These experiments showed that the steady state levels of the mRNAs correlated well with the proteomic data (Fig. 2, S2A). We then searched for putative base-pairings between MicA and the mRNAs whose expression was affected by high MicA expression. Besides the known *omp*X/*omp*A mRNA targets, basepairing interactions were predicted for the 5′ tail of MicA with *mre*B mRNA and *yrb*E/*mla*E mRNA, which lies in the same operon as *yrb*C/*mla*C (Figure S2B–D). Notably, Hfq, the co-factor of many sRNAs in *E. coli*, has been shown to repress the expression of *mreB* at the post-transcriptional level [12]. However, a translational reporter assay performed with the leader region of *mre*B fused to *lac*Z did not show any effect of MicA on protein synthesis (Figure S2C) while a slight but specific repression of MicA on YrbE/MlaE-LacZ synthesis was observed only in cells grown until OD 0.3 (Figure S2D). As a positive control, we showed that MicA strongly repressed the synthesis of OmpX-LacZ under identical experimental conditions (Figure S2B). These data suggested that some of the MicA-dependent effects observed in this study might be indirect (see discussion in Supplementary Information). However, the reporter gene constructs are different from the genomic context of *mre*B and *yrb*E/*mla*E, and we do not exclude that the selected regulatory regions are only partial. Interestingly, the *yrb*/*mla* operon is the ABC transport system that preserves lipid asymmetry in the Gram-negative outer membrane particularly in stressed cells [13]. Hence, MicA might contribute to maintain the lipid asymmetry under membrane stress conditions.

The same approach was performed on the *S. aureus* strain RN6390 and its isogenic Δ*rna*III derivative strain (Fig. 2(B)). The data revealed that the synthesis of protein A, a major cell-surface protein involved in adhesion and innate immune evasion, is repressed by RNAIII in agreement with previous observations that RNAIII binds to *spa* mRNA to inhibit translation [14]. In addition, the levels of several transporters were significantly decreased by RNAIII (Figure S3A). Surprisingly, several enzymes involved in the metabolism, likely localized in the cytoplasm, were found in the Δ*rna*III mutant strain, although these proteins did not vary significantly between both strains in total protein extracts (result not shown). These data suggested that the absence of RNAIII rendered the membrane more permeable to the dye although significant lysis of bacteria was not observed. This is consistent with the fact that the synthesis of several peptidoglycan hydrolases (LytM, Sa2353) is repressed by RNAIII in late exponential growth [15]. Thus, we propose that the absence of RNAIII induces an overproduction of these enzymes, modifying the remodeling of the peptidoglycan and the physical properties of the cell wall.

In conclusion, direct labeling of cell surface/membrane proteins is a sensitive approach to monitor changes of the cell envelope composition governed by regulatory RNAs. The method can easily be applied to monitor the effect of other types of regulators (proteins, metabolites) and to follow dynamical properties of the membranes in response to stress and varying environmental conditions.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2014.07.011.

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