"Protein fingerprinting of *Staphylococcus aureus* by capillary electrophoresis with on-capillary derivatization and laser-induced fluorescence detection"

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Summary

This chapter describes a complete procedure for obtaining protein fingerprints of microorganisms using capillary electrophoresis (CE) with laser induced fluorescence detection (LIF). *Staphylococcus aureus*, a human pathogen responsible of frequent and resistant infections, is used as model microorganism to show the feasibility of this procedure. Bacteria are grown in different culture media or submitted to temperature or nitrosative stress conditions. After the growth of the bacteria, the protein extracts are obtained by cell lysis using sonication. The water-soluble fraction of these lysates is derivatized on-capillary with the fluorogenic reagent 3-(2-furoyl)quinoline-2-carboxaldehyde. The fluorescent products are analyzed by CE and detected by LIF. Practical advices for the interpretation of the electropherograms are given. To do so, the variations of the protein fingerprints of the bacteria with the culture conditions, such as growth medium, or the stressing conditions, such as heat shock or nitrosative stress, are used as example.

Keywords

Capillary electrophoresis, laser-induced fluorescence detection, on-column derivatization, protein fingerprinting, *Staphylococcus aureus,* growth conditions, stressing conditions.

1. Introduction

Capillary electrophoresis (CE) has demonstrated to be a powerful tool, either to separate intact microorganisms ***(1,2)*** or to obtain protein fingerprints of different microorganisms

(3,4). When combined with laser-induced fluorescence detection (LIF), minute amounts of proteins can be analyzed, even at the level of single cells ***(5,6)***. In addition, the development of on-capillary, covalent ***(5,6)*** or non-covalent ***(7,8)*** derivatization methods for CE-LIF allows the reduction of the reaction time and the consumption of reagents, the use of a small sample volume, and automation of the analytical process. Several investigations can be carried out using protein fingerprints of bacteria such as adaptation of microorganisms to modification in their environment, i.e., in their culture media or in their accommodation to the stressing conditions. Furthermore, the coupling of CE techniques with mass spectrometry ***(9)*** has shown to have a great potential in these studies due to the identification capabilities of the MS techniques in protein analysis.

Bacteria are able to rapidly adapt to changing environments. In fact, bacteria deploy only those factors required for growth and survival within a particular environment and they show a number of genetic responses to environmental conditions ***(10)***. In particular, under stress conditions bacterial cells synthesize proteins which are different from those produced under normal conditions. Most of them are highly conserved proteins which appear as response to different stimuli ***(11)***. This is the case of the proteins known as heat shock proteins (hsp) ***(12)*** or those known as cold shock proteins (csp) ***(13)***. For instance, Laport et al. ***(12)*** have reported the synthesis of three new proteins with a molecular weight higher than 90 kDa when the growth temperature of a culture of *Staphylococcus aureus* was increased from 37 to 42 ºC. Also, Richardson et al. ***(14)*** reported the stress responses of *S. aureus* to the nitric oxide produced by mammalian hosts as a defence against the bacteria. The protein maps obtained by two-dimensional gel electrophoresis (2D-PAGE) ***(15)*** or by transcriptomic analysis ***(16)*** of the nitrosative stress response of *S. aureus* have allowed the demonstration of changes

in protein patterns under stressing conditions. However, these techniques are laborious and time-consuming and the protein maps obtained are usually complex. Similarly bacteria can modify the proteins expressed to adapt themselves to modifications in culture media ***(17)***.

Besides, protein fingerprinting obtained by CE-LIF has allowed establishing differences between species that can contribute to their identification and classification ***(18,19)***. Therefore, the identification of bacterial species based on these protein profiles could complement the information obtained by classical microbiological assays.

This chapter reports a detailed procedure for obtaining the protein fingerprinting of *S. aureus* using CE-LIF. The procedure has the following major steps: (1) Bacteria growth at different culture conditions. (2) Protein extraction after cell lysis. (3) On-capillary derivatization of the extracts of proteins with the fluorogenic reagent 3-(2-furoyl)quinoline-2-carboxaldehyde together with the analysis of the fluorescent products by CE-LIF. A section dealing with interpretation of results has been introduced in which the interpretation of the electropherograms of the protein fingerprints obtained by CE-LIF is carried out. In this section, the electropherograms obtained when the culture media are modified and those obtained as a response of the bacteria to stressing conditions (temperature and nitrosative stress) are used as examples. Although in this chapter the procedure is demonstrated to be feasible for *S. aureus*, it can also be used for other microorganisms.

2. Materials

2.1. Bacterial strains and culture media

1. The strain employed was *Staphylococcus aureus* CECT 4465 from the Spanish Type Culture Collection.

2. *S. aureus* was maintained in Nutrient Agar plates (NA) (Pronadinasa, Madrid, Spain).

3. Bacteria were grown in nutrient broth (NB) or tryptic soy broth (TSB) at different times and/or different stressing conditions (temperatures and NO concentration) depending of the investigation carried out (see Methods).

4. The *S. aureus* growth curves were determined by measuring the OD at 600 nm using an S-22 Boeco spectrophotometer (Boeckel, Hamburg, Germany).

5. In nitrosative stress studies of *S. aureus*, the nitric oxide donor diethylammonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate (DEA-NONOate) Alexis Biochemicals (San Diego, CA, USA) was used.

6. To evaluate NO formation in the cultures, the Griess assay was used. For this assay sulfanilamide and N-(1-naphtyl)ethylenediamine dihydrochloride used for the peparation of Griess reagents were obtained from Sigma Chemical (St. Louis, MO, USA) (See Note 1).

2.2. Lysis of bacteria and protein extraction

1. For washing the microorganism prior to lysis, PBS was used (See Note 2).

2. Lysis of bacteria was carried out by ultrasonication using a Misonix (Farmingdale, NY, USA) cell disruptor in 15 mL falcon tubes.

3. Total protein concentration was estimated in the supernatant of the bacterial lysates by the Bio-Rad (Hercules, CA, USA) protein assay based on the Bradford dye-binding procedure ***(20)***.

2.3. On-column derivatization of proteic extracts

1. Labeling reagent was 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ) from Molecular Probes (Eugene, OR, USA) (See Note 3).

2. The nucleophilic agent for the derivatization with FQ was potassium cyanide from Sigma (See Note 4).

2.4. CE-LIF Instrumentation

1. The in house made CE apparatus with LIF detection employed previously described ***(21)*** was used with some modifications ***(22)***. Briefly, an RS/EH50R high voltage power supply (Glassman High Voltage, Whitehouse Station, NJ, USA) was used in normal polarity configuration (anode connected at the inlet end of the capillary). Sample injection was carried out by raising the sample vial at a given high during a given time (injection by siphoning). A 2060-10S Spectra Physics Ar-ion laser (9 mW) (Spectra Physics, Mountain View, CA, USA) was used as an excitation source at 488 nm in LIF with an orthogonal configuration. Fluorescence was collected using a 40x plan-achromatic microscope objective, passed through a 550 nm cut-off filter and an interference filter centered at 590 nm, imaged onto an iris to block straight light, and detected with an R928 photomultiplier tube (Hamamatsu, Hamamatsu City, Japan) operated at 600 V and mounted on top of a high-precision stage for alignment (Newport, Mountain View, CA, USA). Photocurrent was processed by a 7070 photometer (Oriel, Stratford, CT, USA) and a 406 System Gold A/D converter (Beckman, Fullerton, CA, USA). A laboratory-made device was used to heat a small zone (10 cm) at the inlet of the capillary (See Note 5) where the derivatization reaction took place, using an F3 water thermostatic bath (Haake, Karlsruhe, Germany).

2. Uncoated capillaries (Composite Metal Services, Worcester, UK) 60 cm length (50 cm to the detector) and 50 µm I.D. (375 µm O.D.) were used (See Note 6).

3. CE running buffer was 50 mM phosphate, 15 mM sodium pentyl sulfate (SPS) (pH 7.0).

4. For sample solvent preparation a volume of approximately 20 µL of 1 M NaOH was added to 10 mL of the running buffer to make a solution of pH 11.0.

5. A 200 mM KCN stock solution was made in 2.5 mM borax (pH 9.3) (See Note 4).

3. Methods

3.1. Culture conditions

1. For getting the protein fingerprinting of *S. aureus* grown in different culture media (See Note 7).

- Inoculate one or two colonies per tube of *S. aureus* from the agar plate in 16 mm internal diameter tubes containing 5 mL of either TSB or NB media. Incubate for 24 h with orbital shaking at 200 rpm at 20 ºC.
- At the indicated time take a 200 µL aliquot of the culture and perform bacteria lysis and protein extraction.
- Each culture must be done at least in duplicate.

2. For getting the protein fingerprinting of *S. aureus* grown at different temperatures.

- Inoculate one or two colonies per tube of *S. aureus* from the agar plate in 16 mm internal diameter tubes containing 5 mL of TSB.
- Incubate with orbital shaking at 200 rpm for 60 h at 20 ºC and for 15 h at 42 ºC.

- At the indicate time take a 200 μL aliquot of the culture and perform bacteria lysis and protein extraction.

- Each culture must be done at least in duplicate.

3. For getting the protein fingerprinting in nitrosative stress conditions.

- Prepare a preinoculum by inoculating one of two colonies of *S. aureus* from the agar plate in a 16 mm internal diameter tube containing 5 mL of NB media and incubate overnight with orbital shaking at 200 rpm and 20ºC.

- Inoculate 100 μL of this preinoculum in 10 mL of NB culture broth pH 7.3 in 50 mL Erlenmeyer flask and incubate with orbital shaking at 200 rpm and 37 ºC.

- Measure the optical density of the *S. aureus* culture at intervals of 30 min using a spectrophotometer to follow the growth of the *S. aureus* culture (See Note 8).

- When OD_{600nm}~1, that is mid-log phase of *S. aureus* growing (after approximately 4 hours of growing), add to the culture a mixture of 22 μL of 500 mM DEA-NONOate solution (See Note 9). Measure the pH in every culture before adding the NO donor to be certain of working around the physiological pH (See Note 10).

- Take a 200 μL aliquot of the culture 1 h after the addition of the NO donor (approximately after 5 h of growing). Perform bacteria lysis and protein extraction of this aliquot.

- Each experiment must be done at least in duplicate.

3.2. Lysis of bacteria and protein extraction

1. Eliminate the culture medium by centrifugation at 5000 g for 5 min at room temperature.

2. Wash the pellets 3 times in PBS solution and resuspend in Milli-Q water.

3. Disrupt the bacteria on an ice bath by ultrasonication (4x, 60 sec).

4. Centrifuge the crude sonicate at 6000 g for 20 min at room temperature and collect the supernatant.

5. Determine the total protein concentration in an aliquot of each extract by the Bradford dye-binding procedure.

6. Store the rest of the supernatant at -20ºC until performing the analysis by CE-LIF.

3.3. Sample preparation for on-capillary derivatization

1. Thaw the aliquot of protein extract to be studied.

2. Mix it with the corresponding volume of a 200 mM KCN solution, and then dilute it with a 50 mM phosphate, 15 mM SPS (pH 11.0) buffer solution to obtain a final concentration of 5 mM KCN and approximately 5 x 10^{-7} M of total protein in the final mixture of all the extracts studied.

3.4. On-column derivatization of the extracts and CE-LIF analysis

The procedure for on-capillary derivatization is based on a previously optimized method ***(23)***.

1. New capillaries were rinsed with 1 M NaOH (100 µL) followed by a rinse with Milli-Q water (100 µL). Between runs, the capillary was sequentially rinsed with Milli-Q water (100 µL), 0.1 M NaOH (100 µL), Milli-Q water (100 µL), and the separation buffer (100 µL). Rinses were made manually employing a model 1710 glass syringe from Hamilton (Bonaduz, Switzerland).

3. Inject a plug of a mixture of the sample added with KCN solution into the capillary. Inject 5 mM solution of FQ in separation buffer. Both injections are performed by siphoning (20 cm height) for12 s (See Note 11).

4. Mixing step: Place the vials containing the separation buffer in the inlet and outlet ends of the capillary and apply high voltage of 3 kV for 6 min. Perform the step at 65 ºC by using the device described in Note 5.

5. Reaction step: Switch off the power supply for 15 s (note that reaction step is carried out also at 65 ºC).

6. Separation step: Apply 15 kV at room temperature (See Note 12).

7. Collect the electropherograms. Analyze each sample at least in duplicate.

4. Interpretation of results

4.1. Fingerprint repeatability

The repeatability of the protein patterns can be analyzed for injections of the same culture and for different cultures grown under identical conditions. The Figure 1 shows that the protein profiles were very similar, the number and the position of the peaks in the protein patterns were always reproducible, however slight differences in the height of some of the peaks were observed. Therefore, in our experience the peak height cannot be considered as a differentiation factor between conditions. Probably, these differences observed among cultures could be due to variations in bacterial growth and the consequent protein content (compare Figure 1B with Figure 1C, D, or E), even

though the total amount of protein in the different samples was the same (6.4×10^{-7} M according to the Bradford assay). No peaks were observed in the electropherograms of a blank, i.e., culture media where no microorganisms were grown (see Figure 1A).

[Figure 1 near here]

It is worth mentioning that for pattern repeatability it is of special importance to work, whenever possible, with the same fused silica capillary for CE-LIF experiments. Protein fingerprints obtained with other different capillaries from the same brand but different batch are shown in Figure 1F (compare electropherogram in trace F with any of the traces B-E), where it can be observed that migration time of the peaks and the peak heights are different from capillary to capillary. It is well known that small modifications on the EOF of the capillary cause slight differences in the migration time of analytes, thus influencing repeatability. Moreover, we have observed that a capillary that has been used for a large number of runs (>20) is degraded and different migration times are obtained with respect to new capillaries. However the three different regions (indicated as Region 1, 2 and 3 in the figures) of the electropherogram in which the peaks seem to cluster can be easily recognized from capillary to capillary.

4.2. Applications

Using the procedure described in this chapter the effect of different growth conditions of the microorganisms can be studied. As an example, the effect on proteins patterns of *S. aureus* grown in two different culture media, NB and TSB, were compared.

[Fig 2 near here]

Figure 2 shows that the profiles corresponding to both cultures were different. Some peaks could correspond to over-expressed proteins while others are related to infra-expressed proteins. For instance, the profile corresponding to the culture grown in NB presents three peaks in region 3 that are not observed in the profile of the culture grown in TSB; however, a shift in migration time of the peaks probably due to differences in EOF could not be ruled out. In consequence, the method developed allows the differentiation of cultures of *S. aureus* grown in different media on the basis of these protein patterns.

CE-LIF technique can also be used to study proteins fingerprints of microorganisms in different stressing conditions. We have studied ***(19)*** the effect of temperature and NO on the fingerprint of *S. aureus.* As an example of the interpretation of these electrophoretic patterns, we discuss briefly some details on both studies.

To study the temperature effect two cultures of *S. aureus* were grown at 20 and 42 ºC (in TSB medium for 60 and 15 h, respectively). These are suboptimal temperatures for *S. aureus* which has an optimum growth temperature of 37 ºC ***(12)***. In that experiments two different growing times (60 and 15 h) were used to obtain approximately the same number of bacterial cells at the end of the same growing phase of the microorganism than that obtained at the optimal growth temperature. In Figure 3A and B the protein profiles obtained for cultures of this microorganism grown at these two temperatures are shown.

[Figure 3 near here]

Although, several peaks could differentiate both electrophoretic profiles, only the peaks showing major differences will be considered here. For instance, by comparing Figure 3A and B with Figure 3C it can be readily seen that peak 1 is only shown in the profiles of the cultures grown at 42 ºC and peak 5 is the major peak in the cultures at 20 ºC, while it is scarcely present when the microorganism is grown at 42 ºC. Minor peaks, such as peaks 3, 4, and 10, could in addition serve to differentiate both cultures, but these minor components are more sensitive to variations among culture, as indicated before. Temperatures of around 42 ºC are considered extreme for *S. aureus* and it could be hypothesized that these peaks could be related to proteins induced in stress conditions, such as heat shock proteins ***(12)*** or cold shock proteins ***(13)***.

Finally, the effect of the exposure of *S. aureus* to NO on the electrophoretic fingerprint of proteins has been studied. Nitric oxide (NO) is an important antimicrobial agent and it has been shown to be indispensable for the clearance of diverse pathogens. Several studies have suggested that NO is bacteriostatic and bactericidal for *S. aureus* ***(24-26)*** although adaptative response of these bacteria to nitrosative stress is not clear. The optimum growth temperature of the bacteria (37 ºC), which had been grown for 18 h, was selected and the effect of the NO donor DEA-NONOate on the fingerprint of *S. aureus* was investigated. As shown in Figure 4 for cultures grown in the presence of the donor, the height of the peaks marked as 1 and 2 in the electropherogram has increased, however peaks in region 2 of the electropherogram have decreased, or even some peaks are not observed.

[Figure 4 near here]

Therefore, the procedure described allows one to differentiate the profiles *S. aureus* exposed and not exposed to the effect of NO. Transcriptomic analysis of the nitrosative stress response of *S. aureus* has identified both up-regulated and down-regulated genes (66 and 18 genes, respectively) in response to NO-donors ***(16)***.

4. Notes

Note 1. Griess assay ***(27)*** measures the nitrite ion (NO_2^-) concentration, a rapid, stable, and non volatile breakdown product of NO in solution. The test uses two reagents. Reagent 1 consists of a solution of 1% (w/v) of sulfanilamide in 5% (v/v) of phosphoric acid. Reagent 2 is prepared by adding 0.1% (w/v) of N-(1-naphthyl)ethylenediamine dichlorhydrate in Milli-Q water. Griess reagents in presence of nitrite ions produces a diazo compounds (with purple color) whose absorbance can be measured at 540 nm.

Note 2. PBS consists of 0.01 M disodium hydrogenphosphate/sodium dihydrogenphosphate, 0.138 M NaCl, and 2.7 mM KCl (pH 7.4).

Note 3. Preparation of FQ solutions: A 50 mM stock solution of FQ was prepared in methanol (HPLC grade, Scharlau Chemie, Barcelona, Spain). Since FQ degrades in solution even when stored at -20 ºC in darkness, small aliquots of dried FQ were prepared. To do so, 10 µL aliquots of the methanolic solution were transferred to 500 µL microcentrifuge tubes. The solvent was removed under vacuum at room temperature using a model RC10-10 centrifugal evaporator (Jouan, Saint-Herblain, France). The dried FQ aliquots were stored at -20 ºC protected from light until use. On the day of the

experiment, the aliquots were defrosted and solved in the running buffer to obtain a 5 mM FQ solution.

Note 4. Potassium cyanide is highly poisonous. It reacts with acids to form lethal HCN gas. Therefore, stock solutions should be made in a basic buffer. Neutralization of waste containing KCN solution should be done by the addition of 1% NaOH solution (about 50 µL NaOH/g cyanide) followed by slow addition of bleach (about 70 µL bleach/g of cyanide).

Note 5. The heating device was made using two PVDF T-pieces model 1C300T03PV (EM-Technik, Maxdorf, Germany) interconnected by a 1 cm-long Tygon tube (6 mm O.D., 5 mm I.D.) using one entrance of each of the T-pieces. This assembly formed a jacket that allowed the insertion of the first 10 centimeters of the separation capillary through the distal entrances of the interconnected T-pieces. The capillary was retained in the T-pieces by inserting it through silicone septa. This jacket allowed the control of the temperature in this length of the capillary using water from an external circulating thermostatic bath entering the device through Tygon tube connected to the two remaining entrances of the T-pieces. Water-tightness of the device was achieved using PVDF cutting rings and thumb-nuts provided with the T-pieces for Tygon tubing connections and silicone septa used for the insertion of capillary.

Note 6. Capillaries are purchased in reels and cut to the desired length. Detection window is made by burning off the polyimide layer.

Note 7. Although in this chapter the whole procedure is described only for the species *S. aureus*, we have also used it for study other species, such as *Staphylococcus epidermidis* ***(19)***.

Note 8. To asses the effect of the addition of DEA-NONOate on the physiology of the *S. aureus* the growth curves of this microorganism (variation of the OD $_{600nm}$ value of the cultures with time) were determined in nitrosative stress conditions and in the control cultures. To do so, three cultures were grown simultaneously at 37ºC in NB culture media. Culture 1 (supplemented with DEA-NONOate) was prepared as follows: when the $OD_{600\ nm}$ value of the *S. aureus* culture was around 1 (at about 4 h after the start of the culture), it was added with 22 µL of a 500 mM solution of the NO donor in 0.01 M of NaOH in water. Culture 2 (culture supplemented with 0.01 M NaOH) was prepared as Culture 1, but it was added with only 22 µL of an 0.01 M aqueous solution of NaOH (the solvent used for the NO-donor) and no NO donor was added. Culture 3 (untreated culture) was prepared as Cultures 1 and 2, but nothing was added to the culture. In the three types of cultures 100 µL aliquots were sampled at intervals of 30 min since the start of the culture. The samples were diluted (1:4) in Milli-Q water and the OD $_{600nm}$ was determined. An example of the growth curves obtained in these experiments is given in Figure 5. It can be observed that the release of NO in the culture causes a decrease in the growth rate of the microorganism. This alteration can potentially produce a change in protein expression and therefore in the protein fingerprint obtained by CE-LIF.

[Figure 5 near here]

Note 9. In Griess assay, measurements were carried out by duplicate in 96-well plates. In each well 50 μL of sample and 50 μL of the Griess reagent 1 (See Note 1) were added to the well and sample was incubated for 5 min at room temperature in the darkness. Then, 50 μL of the Griess reagent 2 (See Note 1) were added and the solution was incubated again for other 5 min in the same conditions. Finally, the absorbance at 540 nm was measured in a plate reader. Solutions of sodium nitrite in NB solution (concentration range 3-100 μM) placed in other wells of the same plate were used to obtain the calibration curve. The concentration of nitrite ion in the sample was determined by interpolation of the average values of the reading of two samples in the calibration curve. As controls, aliquots containing only the (clean) nutrient broth NB, culture containing bacteria, and NB solution added with NO donor were used. As an example, variation of the NO_2^- present in the cultures in the time elapsed after the addition of the NO donor is given in Figure 6. The addition of 1.1 mM DEA-NONOate, a rapid releasing NO donor, produced a NO_2^- concentration of 1.1 mM in 30 min, that decreased slowly until a concentration of 0.9 mM after 4 h of its addition to the culture As expected, the controls (the clean culture medium, and the bacteria cultures in the absence of NO donors) in two individual assays, did not produce absorbance at 540 nm, what indicates the absence of their interference in Griess assay. The third control, the culture medium in the absence of bacteria supplemented with the NO donor, produced a concentration of NO_2^- higher than the maximum value reached in presence of bacteria when DEA-NONOate was added, probably due to the absence of cells that would react with NO (results not shown in Figure 6)

[Figure 6 near here]

Note 10. This control was performed due to the fact that, although insensitive to most buffer constituents and contaminants, DEA-NONOate decomposition is extremely sensitive to small changes in pH ***(28)***.

Note 11. After each injection of sample plus KCN or FQ, the inlet end of the capillary was washed by immersing it 3 times in a vial containing Milli-Q water to avoid cross-contamination of the sample and the FQ solution.

Note 12. Room temperature was set at 24 ºC.

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Figure captions

Figure 1. Capillary electrophoresis patterns of a blank assay (A), patterns of three consecutive injections of the same culture of *S. aureus* (B–D) and two cultures of *S. aureus* grown in the same conditions (D and E). Growth conditions: temperature 37 ºC, medium NB, and time 18 h. CE-LIF conditions: running buffer 50 mM phosphate, 15 mM SPS (pH 7.0), uncoated capillary (L= 60cm, l = 50 cm, 50 μm I.D.), injection by siphoning (h = 20 cm, 12 s) of a mixture of the protein extract with a 5 mM KCN solution followed by an injection of 5 mM FQ in running buffer, mixing 6 min at 3 kV, reaction 15 s at 0 kV, mixing and reaction temperature 65 ºC, separation performed at 15 kV and room temperature. Trace F represents the electrophoresis pattern of a culture of *S. aureus* obtained in the same condition than traces B-E (for both, culture and the CE-LIF separation) carried out in a new capillary. "Adapted from Analytica Chimica Acta, 659, Veledo M.T., Pelaez-Lorenzo C., Gonzalez R., de Frutos M., Diez-Masa J.C., Protein fingerprinting of *Staphylococcus* species by capillary electrophoresis with

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Figure 2. Capillary electrophoresis patterns of proteins for cultures of *S. aureus* grown in (A) NB medium and (B) TSB medium. Other growth conditions: temperature 20 ºC, time 24 h. Rest of the conditions as in Figure 1. "Reprinted from Analytica Chimica Acta, 659, Veledo M.T., Pelaez-Lorenzo C., Gonzalez R., de Frutos M., Diez-Masa J.C., Protein fingerprinting of *Staphylococcus* species by capillary electrophoresis with on-capillary derivatization and laser-induced fluorescence detection, 81-86, Copyright (2010), with permission from Elsevier."

Figure 3. Capillary electrophoresis patterns of proteins for (A) a culture of *S. aureus* grown at 20 ºC for 60 h, (B) a culture of *S. aureus* grown at 42 ºC for 15 h and (C) a mixture 1:1 of the protein extracts of both cultures shown in (A) and in (B). Medium: TSB. Other conditions as in Figure 1. "Reprinted from Analytica Chimica Acta, 659, Veledo M.T., Pelaez-Lorenzo C., Gonzalez R., de Frutos M., Diez-Masa J.C., Protein fingerprinting of *Staphylococcus* species by capillary electrophoresis with on-capillary derivatization and laser-induced fluorescence detection, 81-86, Copyright (2010), with permission from Elsevier."

Figure 4. Capillary electrophoresis patterns of proteins for (A) a culture of *S. aureus* grown in NB and (B) *S. aureus* grown in NB supplemented with 22 µL of 500 mM DEA-NONOate. Culture conditions 5h at 37 ºC. See subsection 3.1., Culture conditions, for the time at which the NO donor was added. Other conditions as in Figure 1. "Reprinted from Analytica Chimica Acta, 659, Veledo M.T., Pelaez-Lorenzo C.,

Gonzalez R., de Frutos M., Diez-Masa J.C., Protein fingerprinting of *Staphylococcus* species by capillary electrophoresis with on-capillary derivatization and laser-induced fluorescence detection, 81-86, Copyright (2010), with the permission from Elsevier."

Figure 5. *S. aureus* growth curves for the untreated culture (Culture 3 in Note 8), the cultures supplemented with 0.01 M NaOH in water (the solvent of the NO donor) (Culture 2 in Note 8), and culture supplemented with 22 μL of 500 mM DEA-NONOate (Culture 1 in Note 8). The arrow indicates the time at which the NO donor was added.

Figure 6. Measurements by Griess assay of NO production (expressed as NO_2^- concentration in the culture medium) after the addition of the DEA-NONOate to a *S. aureus* culture. The NO donors were added to the cultures at the mid-log phase of the microorganism growing (see arrow in Fig. 5). Measurements were made every 30 min for a 6 h period.