9. BIOLOGICAL MATERIALS AND METHODS

9.1. TEMPLATE DNA

The vast majority of template DNA used for experimentation within this doctoral work was genomic DNA cloned into plasmids. This is due to a number of reasons. Firstly, it is the common practice at the Molecular Microbiology and Bacterial Genetics Group[10], which was the main supplier of the biological material here used. Secondly, genomic DNA cloned into a plasmid is easier to manipulate, since the plasmid can be introduced into a number of culture vectors (*Escherichia coli* or other easy-cultured bacteria) whereas genomic DNA may correspond to an organism of difficult culture (like *Mycobacterium leprae*) or handling (for example, a pathogenic bacterium such as the same *Mycobacterium leprae* or *Neisseria meningitidis*). Additionally, and depending on the vector, more than one plasmid may be inserted into a single bacterium, meaning that the yield in final DNA concentration will be higher than (i.e. a multiple of) that of genomic DNA. Finally, and for reasons previously stated (see p.72), amplification of plasmid DNA is typically easier to carry out and optimize, since template length is shorter and its %G+C content can be readily addressed. The following is a detailed account of the methodology used to generate template plasmid DNA for the subsequent assays with chips.

9.1.1. GENOMIC DNA EXTRACTION

9.1.2. GENOMIC DNA

Four different kinds of genomic DNA were used in this work:

- *Salmonella typhimurium* ATCC14028 strain IS200 (~200 bp) chromosomal region cloned into a pGEM®-T vector.
- *Salmonella typhimurium* ATCC14028 strain *iroN*-promoter chromosomal region cloned into a pGEM®-T vector.
- *Pasteurella multocida* PM25 strain *hfQ-hflX* (~4 kb) chromosomal region cloned into pBluescript II SK (pBSK) phagemid.
- *Xylella fastidiosa* (~700 bp) *lexA* gene promoter chromosomal region cloned into a pGEM®-T vector.

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Genomic DNA amplification

The desired region of genomic DNA was extracted by selective amplification using specific primers from *TIB MolBiol Syntheselabor* (see p.325 for primer selection). PCR was carried out either in a Cetus DNA Thermal Cycler (*Perkin Elmer*) or a Mastercycler personal (*Eppendorf*) thermocycler, with a robust program for efficient genomic amplification (see p.324).

Band recovery

The PCR product (typically between 200-300 bp) was then run on a negative-controlled 0.8-3%-low agarose gel (2.23 g Nursiere Agarose / 0.75 g Agarose in 100 ml of Tris-EDTA 0.5 M / pH 8 10x buffer, with 5 µl ethidium bromide (0.5 µg/ml) as an intercalating agent for UV staining) for 20 min at 110 V (~10 A) and identified under UV light using a ϕ*X174 DNA/HaeIII* ladder (*Promega*). The amplified band was then physically cut under UV light and inserted into previously weight-calibrated eppendorf tubes, not exceeding 0.4 g of gel per tube. After distribution of the recovered band among eppendorf tubes, 300 µl of 6 M NaI were deposited into each tube to dissolve the agarose gel, and all tubes were left floating in hot water (55 °C) until the agarose gel had dissolved. After agarose liquefying, 750 ml of diatomite resin (*Sigma Corp.*) were inserted into each tube, and the mixture was left to rest for 5 min until two physical phases had been formed, thus ensuring that the diatomite resin had captured most DNA. Each eppendorf mixture was then forced through an ionic exchange column (*Promega*). Afterwards, the column was first rinsed with 80% (v/v) isopropanol (*Prancreac*) and centrifuged for 2 min for isopropanol elution. Next, 30 µl of hot (55 °C) milliQ water were deposited onto the column and left to rest during 5 min before elution of water-carried DNA into a new eppendorf tube by 2 min centrifugation at 12,000 rpm in a Sigma 112 centrifuge. Finally, a small sample of the recovered DNA was run on an agarose gel with a ϕ*X174/HaeIII* ladder to evaluate its concentration.

9.1.3. LIGATION

Chromosomal IS200 recovered DNA was ligated to pGEM®-T vector (*Promega*) (or pBluescript II SK, from *GenBank*) using the standard pGEM®-T/pBluescript II kit and bacteriophage T4 ligase under 3:1 (insert:vector) concentration conditions in a 2x Rapid Ligation Buffer (*Promega*). Ligation

was carried on for 2 hours at 4 ºC and then inactivated by incubation at 70 ºC for 10 min and 4 ºC for 3 min.

9.1.4. ELECTRO-TRANSFORMATION

Electro-transformation was carried out to insert exogenous DNA (the pGEM®-T vector containing the IS200 fragment) into competent cells (*Escherichia coli* DH5 strain) following the procedure described by *Dower et al.* [Dower1988]. After culture, 50 µl of *E. coli* DH5 cells were incubated with IS200 DNA for 5 min at 4 ºC. This mix was then deposited into a *Biolabs* 0.2 cm sterile pail that was inserted in a Gene Pulser (*BioRad*) chamber to provide a 5 ms 12.5 kV/cm^2 electric pulse. The short electric pulse disrupted the cell membrane, allowing the insertion of exogenous DNA. Immediately after the pulse, 1 ml of cold BHI (Brain heart infusion, *Oxoid*) was inserted into the bucket to recover the electro-transformed cells, and these were then incubated in a sterile tube for 1 h at 37 ºC prior to culture.

9.1.5. CELL CULTURE

Transformed *E. coli* DH5 cells were cultivated overnight at 37 ºC on a dish containing TF (Terrific broth, [Tartof1987]) with 50 µg/ml Ap (ampicilin) in a non-wet environment. The pGEM®-T vector encodes resistance to ampicilin and thus, the presence of this antibiotic, which is effective against gram-negative and gram-positive bacteria, ensured optimal culture conditions, since only pGEM®-T carrying cells would be enabled to survive. Moreover, the stringency of the medium would also induce *E. coli* cells to favor plasmid expression in order to withstand ampicilin action. After successful pre-culture on dish and checking for contamination, cultivated cells were used for reseeding of a liquid culture, which was cultivated overnight in 10 ml TF with 50 µg/ml Ap (ampicilin) on a Rotabit orbital stirrer (*P-Selecta*) at 200 rpm.

9.1.6. PLASMID DNA EXTRACTION

After liquid culture, plasmid DNA was extracted following the alkaline lysis method of *Sambrook et al.* [Sambrook1989]. Culture liquid was distributed in eppendorf tubes containing 1.5 ml of solution each, and all eppendorfs were centrifuged for 1.5 min at 12,000 rpm. After centrifugation, the overlaying liquid phase was removed using a vacuum pump, leaving the cells in solid phase at the bottom of the tubes. For each eppendorf tube, 100 µl of re-

suspending solution (5% Tris-HCl and 4% EDTA [pH 8, (v/v)] in milliQ water) were deposited into the tube and the solution was re-suspended using a vortex. Afterwards, 200 µl of lysing solution (10% SDS [sodium-dodecyl-sulfate], 2% NaOH [sodium hydroxide] [v/v] in milliQ water) was inserted and left to incubate for 5 min in ice (~4 ºC). After the solution had acquired its predicted mucous texture, 150 µl of precipitation solution (60% potassium acetate [$KC_2H_3O_2$], 11.5% glacial acetic acid [CH_3COOH, *Pancreac*] [v/v] in milliQ water) were added to precipitate solid intracellular material. The remaining liquid transparent phase was centrifuged (and recovered) twice in phenol-chloroform for 5 min for protein extraction. A last rinsing and centrifugation for 5 min with 450 µl of isoamylic chloroform was conducted to eliminate any phenol traces. Finally, the overlying transparent phase was immersed in absolute ethanol [EtOH] to precipitate DNA during 20 min at -20 ºC. The solution was then centrifuged for 5 min and the overlying liquid phase removed prior to lyophilization for 10 min in a DNA Speed Vac (*Savant*) under vacuum at low drying rate. Final re-suspension was carried out at 37 ºC for 20 min in milliQ water with 0.5 mg/ml RNAse, in order to degrade all residual RNA chains beyond sensitive range.

9.2. PCR METHODS

Conventional PCR methods were established both to conduct different experimental assays (like genomic DNA extraction or adsorption assays) and to provide a positive control to on-chip PCR assays.

9.2.1. PCR MIX

Mix preparation

PCR mix elaboration was carried out in a FLV60 laminar airflow chamber (*EuroAire*) equipped with an UV irradiation lamp. The preparatory steps prior to the elaboration of the PCR mix were the following:

- All PCR reusable material (bottles, milliQ water, buckets, etc.) was previously autoclaved in a 118-LRV autoclave (*Matachana*) under the following conditions: 2.2Bar/135 ºC for 15 min, followed by cooling overnight and vacuum negative pressure.

- Reusable PCR material that could not be autoclaved (pipette tip dispensers, polystyrene ice reservoir, etc.), together with non-sterile one-use-only material (ice, gloves, eppendorf tubes) and manual tools (data sheet, pin-ball pen, fountain-pen, etc.), was introduced in the laminar airflow chamber and left to irradiate under UV light for at least 20 min prior to the elaboration of the mix. Eppendorf tubes were labeled before irradiation and were left open during irradiation.

- Aerosol resistant tips (ART) (*Molecular BioProducts*) with a self-sealing barrier were used across all the preparation steps to prevent aerosol-DNA contamination of the tip dispensers.

All PCR preparations included, as a safety measure, one negative control (that is, a tube not containing DNA) to detect contamination and at least one positive control (a segment previously known to amplify under the conditions set) to prevent false negatives. The elaboration of the PCR-mix was completely done on an ice-filled reservoir (~4 °C) to prevent degradation of the reagents and polymerase activity and aerosol resistant tips (ART) (*Molecular BioProducts*) with a self-sealing barrier were used across all the preparation steps to prevent aerosol-DNA contamination of the tip dispensers.

The PCR mix was elaborated using a master mix that was then distributed in 25, 30 or 50 µl tubes prior to the introduction of template DNA. The master mix tube was typically used as the negative control tube. The basic steps in the preparation of the master mix were as follows:

- After the preparatory irradiation, all eppendorf tubes were closed and the following reagents were placed in the laminar flow chamber: PCR buffer, dNTP solution and diluted primers.
- The master mix was elaborated by first depositing the necessary amount of water and subsequently dissolving the appropriate amounts of PCR buffer, dNTP solution and primers.
- After this stage, Taq polymerase was retrieved from the fridge (-20 °C) and carried on ice into the flow chamber to prevent Taq degradation. Environment-exposed gloves were exchanged for sterilized gloves after the introduction of Taq polymerase.
- Taq polymerase was deposited into the master mix, which was then rigorously mixed by repeated suction and ejection with a large pipette

tip. After mixing to homogenously distribute the enzyme and other reagents, the master mix was distributed among the other test tubes in aliquots of 25, 30 or 50 µl. The master mix eppendorf, now negative control mix eppendorf, was closed prior to the introduction of template DNA.

- Template DNA and, when different, positive controls were introduced into each eppendorf tube separately to prevent cross-contamination. As a last preparatory step, if necessary, a homogenous layer of non-irradiated mineral oil was poured into the tubes with a drop dispenser.
- PCR mixes were submitted to a short centrifuge pulse (10 s at 12,000 rpm) before introducing the eppendorf tubes into the thermal cycler.

Mix composition

The basic PCR mix for a final volume of 25 µl (which was afterwards optimized for different templates and PCR-chip conditions) was the following:

17 µl	milliQ water
2.5 µl	10x Taq-buffer (*Roche*) [750 mM Tris-HCl (pH 8.8 at 25 °C), $(NH_4)_2SO_4$, 0.1% - Tween-20, 15 mM $MgCl_2$]
2.5 µl	10x dNTPs 100 mM each (*Roche*)
1.25 µl	Up primer
1.25 µl	Down primer
0.2 µl	Taq polymerase (*Roche*) ~0.7 U
1 µl	30-60 ng/µl template DNA

9.2.2. THERMOCYCLER PARAMETERS

The reactions were carried out either in a Cetus DNA Thermal Cycler (*Perkin Elmer*) or a Mastercycler personal (*Eppendorf*) thermocycler. In case the former was used, non-irradiated mineral oil was poured onto each PCR mix to prevent evaporation and also onto the thermocycler block reservoirs to ensure efficient heat transfer between block and tubes. PCR protocols varied depending on the nature of the template and amplicate DNA, the most manifest of these changes being the variations in annealing temperatures. Nevertheless, extension temperature was maintained at 72 ºC across all experiments and the general rule of thumb: 1 kb per

minute of extension (see p.81) was also usually followed. Regarding denaturation temperatures, 95 ºC was the most frequent choice, although denaturation temperatures of 95.5 and 96 ºC were also used as reference for protocols implemented with PCR-chips. In non-optimized PCR amplifications, the preheating (2 min) and final extension (7 min) steps were always maintained.

9.2.3. PRIMER SELECTION

Primer selection was performed using the application-specific software PrimerSelect 4.0.5 (*DNASTAR*®). PrimerSelect allows the user to introduce a desired length of primers, the desired amplicate region and size, and then scans the sequence file of template DNA searching for suitable primers (see Figure 180 for an instance of program output). For selection of optimal primer-pairs, the program takes into account, among other parameters, primer melting temperature, relative free structural energy and possible occurrence of primer-dimers and self-dimers.

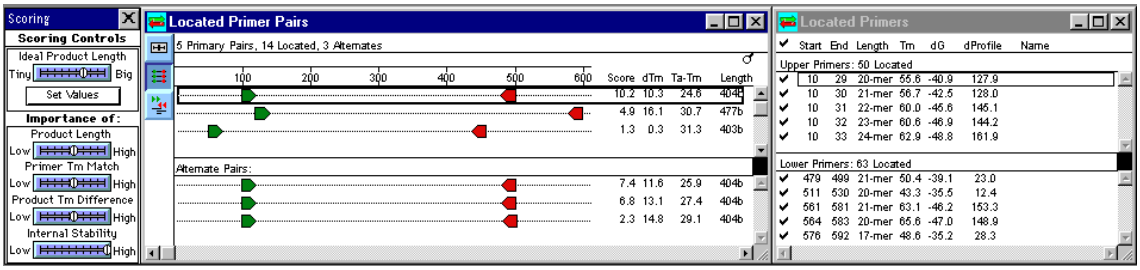

Figure 180 - PrimerSelect output for *E. coli* lexA gene sequence and 10 bp primers; scoring controls on the left provide result-sorting options.

9.3. SLAB GEL ELECTROPHORESIS

Slab gel electrophoresis was conducted both to evaluate PCR results and to recover DNA bands (see p.320 for details). The agarose percentage varied according to fragment size [Maniatis1982], but was usually kept between 0.8 and 3%.

9.3.1. SLAB MAKING

Agarose gel was created by depositing 0.8-3% (w/v) on a tube containing Tris-EDTA 0.5 M / pH 8 10x buffer. The mixture was repeatedly heated to boiling point in a microwave oven, until non-homogeneities were completely

removed. Ethidium bromide (0.5 μg/ml) was then added as an intercalating agent for UV staining and the mixture was left to cool in electrophoresis casting-trays (*BioRad MiniSub® Cell GT*). Wells for DNA insertion were molded into the cooling gel by means of a comb provided by the casting-tray manufacturer. After gel hardening, the comb was removed to expose the wells and the slab was immersed in 10x Tris-EDTA buffer.

9.3.2. GEL LOADING AND RUN

DNA samples were mixed with 10x loading dye (20% Ficoll 400 [*Sigma*], 0.1 M Na_2EDTA, pH 8, 1% sodium dodecyl sulfate, 0.25% Bromphenol blue [*Pancreac*], 0.25% Xylene Cyanol [*Clonetech*]) in 1:10 (dye:DNA) concentration and loaded into the wells. After loading, the gel was run at 110 V (~10 A) for 10-20 min using a BioRad 200/2.0 power supply.

9.3.3. INSPECTION AND EVALUATION

DNA bands were visually inspected using an UVP-TM36 UV transilluminator and often photographed for record with the UVP transilluminator camera and a Mitsubishi Video-Copy Processor P68E. For further quantitative analysis, the gels were scanned with a Kodak Electrophoresis Documentation and Analysis System DC120 camera and analyzed with Kodak Digital Science 1D v3.0.0 analysis software, which is able to detect lanes and bands (or specific regions of interest, ROIs) and extract quantitative information (background fluorescence level, mean lane fluorescence level, mean lane area, etc.) about them. phiX174 DNA/Hae III (*Promega*) and λ-DNA Hind III (*Roche*) were used as ladders for fragment identification and as concentration normalizers in quantitative assays.