Resistance to Antimicrobial Peptides and Stress Response in *Mycoplasma pulmonis*

Lina Fassi Fehri,^{1,2} Pascal Sirand-Pugnet,¹ Géraldine Gourgues,¹ Gwenaél Jan,³
Henri Wróblewski,² and Alain Blanchard^{1*}

INRA Université de Bordeaux 2, UMR Génomique Développement Pouvoir Pathogène, 71 avenue Edouard Bourloux,
33883 Villenave D'Ornon, France¹; Université de Rennes I, UMR CNRS 6026, Campus de Beaulieu,
35042 Rennes, France²; and UMR 1253 STLO, INRA Agrocampus, 65 rue de Saint-Brieuc,
35042 Rennes, France³

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Antimicrobial peptides are widely distributed in nature, and in vertebrates, they play a key function in the innate immune defense system. It is generally agreed that these molecules may provide new antibiotics with therapeutic value. However, there are still many unsolved questions regarding the mechanisms underlying their antimicrobial activity as well as the mechanisms of resistance evolved by microorganisms against these molecules. The second point was addressed in this study. After determining the activity of 10 antimicrobial peptides against *Mycoplasma pulmonis*, a murine respiratory pathogen, the development of resistance was investigated. Following in vitro selection using subinhibitory concentrations of peptides, clones of this bacterium showing increased resistance to melittin or gramicidin D were obtained. For some of the clones, a cross-resistance was observed between these two peptides, in spite of their deep structural differences, and also with tetracycline. A proteomic analysis suggested that the stress response in these clones was constitutively activated, and this was confirmed by finding mutations in the *hrcA* gene; in mycoplasmas, bacteria which lack alternative sigma factors, the HrcA protein is supposed to play a key role as a negative regulator of heat shock proteins. By complementation of the *hrcA* mutants with the wild-type gene, the initial MICs of melittin and gramicidin D decreased to values close to the initial ones. This indicates that the resistance of *M. pulmonis* to these two antimicrobial peptides could result from a stress response involving HrcA-regulated genes.

The innate immune system is the first host barrier against microbial infections in vertebrates. This system is of utmost importance for the protection of the host's epidermis and mucosal surfaces because of their direct exposure to the external environment. A failure in the integrity of these surfaces may allow pathogens to disseminate within their host by invading different tissues and organs and possibly the bloodstream. Production of antimicrobial peptides represents one of the most important arms of the innate defenses (for a review see reference 28); this is particularly true for the respiratory tract because it is permanently exposed to the bacteria which are inhaled (2, 21, 49). In the airways, antimicrobial peptides act additionally as regulators of acquired immunity (for a review see reference 2).

The finding that antimicrobial peptides are key factors in the fight against many pathogens, not only in humans but also in most animal species, suggests that they might be a source of novel antibiotics of therapeutic value. Some success has indeed already been obtained with a number of molecules, reaching the phases of clinical evaluation and even marketing (1, 9, 29). Antimicrobial peptides generally show a wide activity spectrum against bacteria, but they also exhibit some degree of toxicity for the host's cells. Although they are very diverse in structure, origin, and biosynthesis, they share a high affinity for biological

membranes which are, for most of them, the main bacterial target. The disruption of membrane integrity often leads to a rapid death of bacteria because this action is faster than the setup of most resistance mechanisms (for a review see reference 27). Although antimicrobial peptides offer a strong barrier against infection, some bacteria have developed strategies to circumvent this line of defense. The molecular mechanisms underlying this resistance (for reviews see references 16 and 38) include the production of proteases (44) and efflux mechanisms (5, 50) and the modulation of the cell surface electrical charge by modifying envelope components, such as the lipopolysaccharides (19, 22, 26) or the capsule (11).

Several mycoplasmas, bacteria belonging to the class *Mollicutes*, are responsible for infections in animals and humans which are usually progressive and chronic (for reviews see references 8 and 20). Even after the clinical manifestations of the disease have receded, mycoplasmas have been shown to persist in their hosts for extended periods of time (25, 47). Mycoplasmas usually parasitize mucosal surfaces, such as the respiratory and urogenital tracts of their animal hosts, and consequently have to cope with the innate immune system, including the antimicrobial peptides. It has been shown that innate immunity provides an effective defense of the lungs against *Mycoplasma pulmonis*, the etiologic agent of murine respiratory infections (12, 57). In particular, the marked difference in susceptibility to *M. pulmonis* disease between C3H and C57BL/6 mice has been linked to the lesser ability of C3H mice to clear the mycoplasma in the early phase of infection, before the development of adaptive immunity (12).

We have chosen *M. pulmonis* as a model for studying the

* Corresponding author. Mailing address: INRA Université de Bordeaux 2, UMR Génomique Développement Pouvoir Pathogène, 71 avenue Edouard Bourloux, BP 81, 33883 Villenave D'Ornon, France. Phone: (33) 5 57 12 23 93. Fax: (33) 5 57 12 23 69. E-mail: ablancha@bordeaux.inra.fr.

TABLE 1. Antimicrobial peptides used in this study

Peptide ^a	Biological source	Biosynthesis mode ^b	Length (residues)	Type	MIC (μ M)/(μ g/ml) ^c
Dermaseptin B5	Amphibian (<i>Phyllomedusa bicolor</i>)	R	26	Linear peptide	100/263.2
Melittin	Invertebrate (<i>Apis mellifera</i>)	R	26	Linear peptide	3.12/8.8
Alamethicin	Fungus (<i>Trichoderma viride</i>)	NR	20	Linear peptide	6.25/12.2
Gramicidin D	Bacterium (<i>Bacillus brevis</i>)	NR	15	Linear peptide	0.0015/0.003
Gramicidin S	Bacterium (<i>Bacillus brevis</i>)	NR	10	Cyclic peptide	3.12/3.8
Globomycin	Bacterium (<i>Streptomyces</i> sp.)	NR	5	Cyclic lipopeptide	0.8/0.052
Iturin A	Bacterium (<i>Bacillus brevis</i>)	NR	8	Cyclic lipopeptide	25/26.2
Polymyxin B	Bacterium (<i>Bacillus polymyxa</i>)	NR	10	Cyclic lipopeptide	>100/>138.5
Polymyxin E	Bacterium (<i>Bacillus polymyxa</i>)	NR	10	Cyclic lipopeptide	>100/>120
Surfactin	Bacterium (<i>Bacillus subtilis</i>)	NR	8	Cyclic lipopeptide	25/26

^a All peptides were purchased from Sigma Aldrich except dermaseptin B5 and globomycin, which were gifts from Pierre Nicolas (Institut Jacques Monod, Université de Paris 6) and from the Sankyo Company (Tokyo, Japan), respectively.

^b NR, nonribosomal; R, ribosomal.

^c MICs are given for *M. pulmonis* MpUR1.1.

interaction between mycoplasmas and antibacterial peptides because (i) it is a natural respiratory pathogen, (ii) its genome has been sequenced (13), and (iii) it is amenable to genetic manipulation (15). The goal of this study was to determine whether this mycoplasma could develop a resistance to antimicrobial peptides and, if so, to elucidate the underlying molecular mechanism.

After determining the MICs of 10 antimicrobial peptides for *M. pulmonis*, clones of this mycoplasma showing increased resistance to melittin and gramicidin D were obtained after *in vitro* selection. The MIC of tetracycline for these clones was also increased. A proteomic analysis suggested that the stress response in these clones was constitutively activated. This activation resulted from mutations in the *hrcA* gene, which encodes a negative regulator of heat shock proteins; this was confirmed by complementation of the mutants with the wild-type *hrcA* gene.

MATERIALS AND METHODS

Antimicrobial peptides and antibiotics. The origin of the antimicrobial peptides used in this study is indicated in Table 1. Other antibiotics used in this study included tetracycline, doxycycline, chloramphenicol purchased from Sigma Aldrich, and enrofloxacin from Bayer.

Bacterial strains and culture medium. The *M. pulmonis* MpUR1.1 clone was obtained by randomly picking a colony from a culture of the *M. pulmonis* UAB CTIP isolate (13). The mycoplasmas were grown in Hayflick medium containing 5% horse serum unless otherwise indicated. During serial passages, the inoculum was diluted 10 times with fresh medium. For agar plates, Noble agar (DIFCO) at a final concentration of 1% (wt/vol) was added.

For DNA cloning procedures, the *Escherichia coli* strain DH10B [*F*[−] *mcrA* Δ (*mrr-hsdRMS-mcrBC*) Φ 80/*lacZ* Δ M15 Δ *lacX74* *deoR* *recA1* *endA1* *araD139* Δ (*ara* *leu*)7697 *galU* *galK* *rpsL* *nupG*] (Life Technologies) was used. This strain was grown in LB medium, and when needed, the following antibiotics were added: ampicillin (100 μ g/ml) and tetracycline (5 μ g/ml).

Determination of MICs. The antimycoplasma activities of the antimicrobial peptides and other antibiotics were determined as described previously (6, 33). Briefly, mycoplasmas at an initial concentration of 10⁶ CFU/ml were grown in 96-well plates in the presence of twofold serial dilutions of the antibiotics. A change in the color of the phenol red added to the medium as a pH indicator was used to monitor bacterial growth. The MIC was defined as the lowest antibiotic concentration that completely inhibited the growth of the mycoplasma after 48 h.

Selection of clones with increased resistance to antimicrobial peptides. The cultures were performed either in liquid medium or on agar plates. For the selection in broth medium, the first passage was performed with the peptide added at a concentration equal to half the MIC (MIC/2), and this concentration was serially increased by twofold in the next passages. Following this process of selection, individual clones were picked up after plating on agar plates which did not contain the selecting peptide. For the selection on agar plates, a Whatman

disk (diameter, 5 mm) was laid at the surface of the medium, and the peptide (100 μ l) was added directly to it at the concentration of MIC/2 for the first passage as detailed above.

Proteome analyses. Mycoplasmas were collected by centrifugation at the end of the exponential growth phase. Mycoplasma proteins were extracted by two different methods. In method A, the pelleted cells were directly lysed in a solution of 1× phosphate-buffered saline containing 0.5% sodium dodecyl sulfate, and the lysate was brought to 65°C for 15 min. After cooling, 4 volumes of cold acetone was added, and the proteins were recovered by centrifugation (12,000 × g, 15 min, 4°C). The pellet was air dried, and the proteins were solubilized in a cocktail buffer containing 7 M urea, 2 M thiourea, 2% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 1% (wt/vol) dithiothreitol, and 2% (vol/vol) Ampholytes (pH range, 3 to 10; Bio-Rad). In method B, the proteins were directly extracted from the mycoplasma cellular pellet in the same cocktail buffer, except that 2 mM protease inhibitor Pefabloc SC (Roche) was added. The subsequent analytical two-dimensional (2-D) gel electrophoreses were performed as described by others (41). Protein was determined using a modified Bradford assay (39). In the first dimension the isoelectric focusing (IEF) was performed using 24-cm ReadyStrip immobilized pH gradient strips with a nonlinear pH gradient of 3 to 10 (Bio-Rad). The proteins were separated by electrophoresis in the chamber of a PROTEAN IEF cell (Bio-Rad) for a total of 60 to 80 kVh. The second dimension was vertical sodium dodecyl sulfate gel electrophoresis using the buffer system of Laemmli in 12% polyacrylamide gels. After electrophoresis, the proteins were detected either by silver staining (34) or by Coomassie blue R-250 staining. Acquisition of gel images was performed using a GS-800 scanning densitometer and the dedicated PDQuest software (Bio-Rad).

Identification of proteins by mass spectroscopy. (i) In-gel protein digestion. Spots containing the proteins of interest were excised from the gel. When needed, silver-stained spots were destained using the PROTSIL2 Silver kit (Sigma Aldrich) according to the manufacturer's instructions. Spot-containing gel pieces were successively washed in H₂O/methanol/acetic acid (47.5:47.5:5) and in acetonitrile (ACN), dried under a vacuum, rehydrated in 50 mM NH₄HCO₃ containing 10 ng μ l^{−1} trypsin (Sigma Aldrich), and incubated for 5 h at 37°C. This first supernatant was collected and stored at −20°C. The peptides left in the gel pieces were extracted once with 50 mM NH₄HCO₃ and twice with a H₂O/ACN/HCOOH (47.5:47.5:5) solution. These three supernatants were pooled to the first one, concentrated in a vacuum centrifuge to a final volume of 30 μ l, acidified by adding 1.8 μ l of acetic acid, and stored at −20°C. The peptide mixtures were analyzed either by matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS) or by high-performance liquid chromatography–nanospray ion trap tandem MS analyses (LC-MS/MS) (see below), as indicated.

(ii) MALDI-TOF. The MALDI-TOF analysis was performed using a spectrophotometer from Applied Biosystems equipped with a nitrogen laser (337 nm, 20 Hz). The spectra of peptide masses were acquired in “reflectron” mode with a delay of extraction of 130 ns and a matrix of α -cyano-4-hydroxy cinnamic acid (CHCA). The data were collected by gating the masses from 500 to 5,000 Da. The resulting peptide fingerprints were compared to those deduced from the SWISS-PROT databank and from the *M. pulmonis* proteome predicted from its sequenced genome (13) using the MS-Fit tool of the Protein Prospector software

(<http://prospector.ucsf.edu/>). The precision requirement was 30 ppm. The minimum number of peptides required for identification was four for each protein.

(iii) Online capillary LC-MS/MS. The peptide mixture resulting from trypsin digestion was analyzed by online capillary high-performance liquid chromatography (LC Packings) coupled to a nanospray LCQ ion trap mass spectrometer (ThermoFinnigan). Peptides were separated on a 75- μ m (inner diameter) by 15-cm C₁₈ PepMap column (LC Packings). The flow rate was 200 nl/min, and peptides were eluted using a 5 to 50% linear gradient of solvent B in solvent A; solvent A was 0.1% (vol/vol) formic acid in 5% ACN, and solvent B was 0.1% formic acid in 80% ACN. The mass spectrometer was operated in positive-ion mode at a 1.8-kV needle voltage and a 38-V capillary voltage. Data acquisition was performed in a data-dependent mode consisting of a full-scan MS over the range m/z 300 to 2,000, followed by three MS/MS on the three most intense ions in the survey scan in an exclusion dynamic mode. MS/MS data were acquired using a 2- m/z unit ion isolation window, 35% relative collision energy, and a 5-min dynamic exclusion duration. Data were searched against the theoretical proteome of *M. pulmonis* by using the SEQUEST software (ThermoFinnigan).

Recombinant DNA techniques. *M. pulmonis* genomic DNA was extracted using the Wizard genomic DNA purification kit (Promega) according to the supplier's recommendations. The *hrcA* gene (mnemonic Mypu_1420 [13]) from the different *M. pulmonis* clones was PCR amplified using the *hrcAL* (5'TTGG AAAATGGTTTATTTATTTCTCA3') and *hrcAR* (5'TCATCAAAGGCAG AGATGTCA3') primers. The PCR mixture was subjected to denaturation for 2 min at 94°C followed by 40 thermal cycles at 94°C (30 s), 68°C (30 s), and 72°C (2 min), followed by a final 5-min extension time at 72°C. The PCR products were sent to a commercial sequencing company (GENOME Express) for direct sequencing using the dideoxy chain termination method.

The pMPO5 plasmid (6,060 bp) which was previously described (15) harbors the *oriC* region of *M. pulmonis* and the *tetM* gene from the transposon Tn916. The full *hrcA* gene together with 280 nucleotides located upstream from its coding sequence was PCR amplified from the *M. pulmonis* MpUR1.1 genome using the *hrcA1* (5'CCCACATGTATGGAGCAACATAGACCTTCA3') and *hrcA2* (5'CCCACATGTTCATCAAAGGCAGAGATGTCA3') primers that include an *Afl*III site (underlined). The PCR product was digested with the restriction enzyme *Afl*III and inserted in the pMPO5 plasmid digested with the same enzyme to obtain the pHRCA plasmid. The integrity of the *hrcA* cloned gene was verified by sequencing. The MpUR1.1, G1, and M1 clones of *M. pulmonis* were transformed with the pHRCA plasmid in the presence of polyethylene glycol as previously described (15). The transformants were selected in liquid broth containing 5 μ g/ml of tetracycline, and this concentration was increased to 20 μ g/ml in the following passages.

Bioinformatics. The nucleotide and protein sequences were aligned using the ClustalW program (48). The CIRCE putative elements in the genome of *M. pulmonis* were searched using the SPAT program (search pattern annotation tool) available in the MolliGen database (4). The query sequence used was the following CIRCE consensus sequence (56): TTAGCACTC-N₆-GAGTGCTAA, where N represents any nucleotide. A maximum of three substitutions was authorized.

RESULTS

Selection of a set of antimicrobial peptides with activity against *M. pulmonis*. We chose 10 antimicrobial peptides displaying activity against different bacteria, including mycoplasma species (6, 33). This set partially reflected the structural diversity found in nature for these molecules (Table 1). The susceptibility of *M. pulmonis* toward these peptides was assessed by measuring their growth inhibition activity. Collectively, the MICs ranged from 0.0015 to over 100 μ M (Table 1). Specifically, the following order of decreasing efficacy was recorded: gramicidin D (0.0015 μ M) > globomycin (0.8 μ M) > melittin and gramicidin S (3.12 μ M) > alamethicin (6.25 μ M) > iturin A and surfactin (25 μ M) > dermaseptin B5, polymyxin B, and polymyxin E (>100 μ M). Owing to their inactivity toward *M. pulmonis* in the conditions of this experiment, dermaseptin B5 and the two polymyxins were excluded from the rest of the study. This lack of susceptibility to poly-

myxin B is shared by other mycoplasmas, including *Mycoplasma gallisepticum* and *Mycoplasma genitalium* (6).

Selection of *M. pulmonis* clones showing increased resistance to antimicrobial peptides. In order to obtain *M. pulmonis* clones showing increased resistance to at least some of these peptides, the mycoplasma was grown in the presence of increasing concentrations of peptide, starting with a concentration equal to half the MIC. These cultures were performed either in liquid medium or on agar plates.

Although repeated attempts were made, resistant clones were obtained with only melittin and gramicidin D, out of the seven peptides selected for these experiments. With melittin, four clones obtained from two independent rounds of selection in liquid medium (two clones from each round) and one clone obtained by selection on agar plates were kept for further study. With gramicidin D, eight clones obtained from two independent rounds of selection in liquid medium (four clones from each round) and two clones obtained by selection on agar plates were kept for further characterization (Table 2). The MICs of gramicidin D were increased from 16- to 128-fold for the gramicidin-resistant clones, and the MICs of melittin from 2.5- to 4-fold for the melittin-resistant clones. Once obtained, the level of resistance was quite stable without the need to maintain selection pressure (i.e., peptide added to the culture medium). Indeed, after 12 passages of the G1, G8, M1, and M2 clones in liquid medium without peptide, the MICs of melittin and gramicidin D were identical to those before passaging. This stability of the phenotype suggested that the resistance for the peptides was due to a mutation rather than to transient regulation of gene expression.

Cross-resistance of selected *M. pulmonis* clones with other antibiotics. To assess whether or not this resistance to a specific antimicrobial peptide was associated with increased resistance to other peptides, MICs for the resistant clones were determined (Table 2). The clones selected in the presence of melittin (M clones) showed an increased MIC (8- to 32-fold) of gramicidin D. Similarly, among the 10 clones selected in the presence of gramicidin D (G clones), 8 of them showed an increased MIC (4- to 16-fold) of melittin. It should be noted that this cross-resistance was not directly correlated to the level of resistance obtained with the peptide used in the selection procedure. As a matter of fact, two of the G clones, G1 and G3, showing the highest increased MIC of gramicidin D did not show an increased MIC of melittin. No cross-resistance was found for peptides other than melittin and gramicidin D.

The MICs of conventional antibiotics known to be active against *M. pulmonis* were also determined for the peptide-resistant clones and were compared with that for *M. pulmonis* MpUR1.1 (Table 2). Although the MICs of three antibiotics (enrofloxacin, chloramphenicol, and doxycycline) remained unchanged, a 4- to 32-fold increase in the MIC of tetracycline was measured for all the resistant clones (Table 2). This increase was not directly related to the level of resistance to the peptide, neither for gramicidin D nor for melittin.

The level of cross-resistance to gramicidin D, melittin, or tetracycline of the different clones was stable and was not affected by passages in a culture medium without selecting peptide added.

Comparative analyses of proteomes of *M. pulmonis* clones. In order to find the molecular mechanism underlying the an-

TABLE 2. Increased antibiotic resistance of *M. pulmonis* clones selected in the presence of melittin and gramicidin D^a

Selection agent or parameter	Selection medium	Clone	Increase in MIC of antimicrobial agent:									
			Mel	Gd	Ala	Gs	Itu	Sur	EFX	CHL	TET	DOX
Melittin	Liquid medium	M1	×4	×32	—	—	—	—	—	—	×32	—
		M2	×2.5	×32	—	—	—	—	—	—	×4	—
		M3	×2.5	×16	—	—	—	—	—	—	×8	—
		M4	×4	×16	—	—	—	—	—	—	×16	—
	Agar plates	M5	×4	×8	—	—	—	—	—	—	×16	—
Gramicidin D	Liquid medium	G1	—	×128	—	—	—	—	—	—	×8	—
		G2	×8	×128	—	—	—	—	—	—	×16	—
		G3	—	×128	—	—	—	—	—	—	×8	—
		G4	×16	×128	—	—	—	—	—	—	×16	—
		G5	×4	×64	—	—	—	—	—	—	×4	—
		G6	×4	×64	—	—	—	—	—	—	×8	—
		G7	×8	×16	—	—	—	—	—	—	×8	—
		G8	×8	×32	—	—	—	—	—	—	×8	—
	Agar plates	G9	×8	×64	—	—	—	—	—	—	×8	—
		G10	×4	×16	—	—	—	—	—	—	×8	—

^a Fifteen *M. pulmonis* clones (M1 to M5 and G1 to G10) were obtained after multiple rounds of selection in the presence of increasing concentrations of melittin or gramicidin D. This table indicates the increases in MIC for antimicrobial peptides (Mel, melittin; Gd, gramicidin D; Ala, alamethicin; Gs, gramicidin S; Itu, iturin A; and Sur, surfactin) and conventional antibiotics (EFX, enrofloxacin; CHL, chloramphenicol; TET, tetracycline, and DOX, doxycycline). A dash indicates that the MIC was the same as for *M. pulmonis* MpUR1.1. The initial MIC of each antimicrobial peptide for *M. pulmonis* MpUR1.1 is indicated in Table 1. The initial MICs of EFX, CHL, TET, and DOX were 0.4 μ M (0.14 μ g/ml), 6.25 μ M (2 μ g/ml), 0.1 μ M (0.05 μ g/ml), and 0.2 μ M (0.1 μ g/ml), respectively.

tibiotic resistance detected in the G and M clones, we chose a global method of exploration. The proteomic technology was chosen since it was shown to be suitable to elucidate the cellular response of bacteria to antibiotics (3). The proteomes of *M. pulmonis* MpUR1.1 and of G and M clones were analyzed by 2-D gel electrophoresis. Cell proteins were resolved in more than 400 spots after silver staining (Fig. 1A). The proteomes of the G8 and M1 clones were highly similar to each other (Fig. 1B and C) and to the other G and M clones (data not shown). However, they contained several spots with higher intensity than in the reference MpUR1.1 clone (Fig. 1). To identify these upregulated proteins, the corresponding spots were excised from the gels and trypsin digested, and a peptide spectrum was generated by MALDI-TOF MS. The identification of the proteins was performed by peptide mass fingerprinting. The proteins that were upregulated in each of the G and M clones included the heat shock protein DnaK, elongation factor Tu (EF-Tu), enzyme I of the phosphoenolpyruvate transferase system, the pyruvate dehydrogenase alpha and beta subunits, the DNA-directed RNA polymerase alpha subunit, and subunit R3 of a restriction modification system. Although the stress response in *M. pulmonis* has not yet been studied, the upregulation of DnaK suggests that this response could be constitutively activated in the G and M clones. Although the other upregulated proteins are not considered heat shock proteins, some of them have been found upregulated in other bacteria responding to stress conditions. Indeed, in the gram-positive bacterium *Listeria monocytogenes*, there was increased production of elongation factor Tu, mannose-specific phosphotransferase system enzyme IIAB, and pyruvate dehydrogenase after a salt stress (18).

Consequently, to try to discover the genetic mechanism which was responsible for the phenotype of the G and M clones, the elements which regulate the stress response in *M. pulmonis* were investigated.

HrcA and stress response in *M. pulmonis*. In most bacteria, the stress response is mediated by alternative sigma factors and specific regulators. In mycoplasmas, including *M. pulmonis*, genome analysis has revealed only one sigma factor (13), which is homologous to general sigma factor A (or sigma-43 factor) of *Bacillus subtilis*. Therefore, since this work and other reports (52) indicate that mycoplasmas are capable of developing a stress response leading to the upregulation of heat shock proteins, regulatory factors other than alternative sigma factors have to be implicated in the expression of these proteins. The repressor HrcA, which is a regulator of stress response found in numerous bacteria (32, 56), has been predicted to be encoded in all of the 11 mollicute genomes sequenced at this time, and we could confirm this by searching the database MolliGen, which is dedicated to the genomics of *Mollicutes* for HrcA homologues (4; <http://cbi.labri.fr/outils/mollogen/>). Transcriptional studies of the heat shock response in *Mycoplasma pneumoniae* (52) and in silico prediction suggested that the CIRCE-HrcA system could play a role in the response of mycoplasmas to this stress. From these data, it was hypothesized that a mutation in the *hrcA* gene could have occurred in the genomes of the G and M clones.

Therefore, the *hrcA* genes from *M. pulmonis* MpUR1.1 and from eight selected clones (G1, G2, G4, G8, G9, M1, M2, and M5) were PCR amplified and sequenced. The coding and the adjacent intergenic sequences of the *hrcA* gene from *M. pulmonis* MpUR1.1 were identical to the corresponding region in the published genome sequence of the *M. pulmonis* UAB CTIP strain (Fig. 2). In contrast, in all the *hrcA* sequences from the other clones, mutations which would result in a truncated HrcA product were found. With the exception of the G4 and M2 clones, there were insertions localized within a stretch of 29 nucleotides between positions 692 and 721 in the *hrcA* coding sequences of all the clones (Fig. 2). The G1, G8, M1, and M5 clones, which were independently obtained, shared the

G

Spot	Mypu no	Identification	Matching peptides	
			Matching / Total	% sequence coverage
a	Mypu_2230	Heat shock protein DnaK	20 / 206	41
b	Mypu_6020	Phosphoenolpyruvate protein phosphotransferase (enzyme I)	16 / 220	35
c	Mypu_0780	Restriction-modification enzyme, subunit R3	8 / 119	20
d	Mypu_7630	Pyruvate dehydrogenase E1 component, β subunit	22 / 183	66
e	Mypu_4050	Elongation factor Tu (EF-TU)	23 / 178	70
f	Mypu_7640	Pyruvate dehydrogenase E1 component, α subunit	21 / 220	52
g	Mypu_5610	DNA dependent RNA polymerase, α subunit	23 / 160	77

FIG. 1. Comparative proteomic analysis of *M. pulmonis* MpUR1.1 (A), the G8 clone (B), and the M1 clone (C). Proteins were extracted from mycoplasma cells using method A (see Materials and Methods). Panels D, E, and F are enlargements of selected regions from panels A, B, and C, respectively. Two-dimensional electrophoresis was performed using 24-cm strips with a nonlinear pH gradient of 3 to 10 in the first dimension and a 12% polyacrylamide gel in the second dimension. Proteins were detected by silver staining. Molecular masses are given in kDa. Seven upregulated proteins were identified by mass spectrometry (MALDI-TOF): DnaK (a), enzyme I of the phosphoenolpyruvate transferase system (b), subunit R3 of a restriction modification system (c), a pyruvate dehydrogenase beta subunit (d), EF-Tu (e), a pyruvate dehydrogenase alpha subunit (f), and a DNA-dependent RNA polymerase alpha subunit (g). (G) The corresponding gene (Mypu_no) is indicated according to the published nomenclature of the *M. pulmonis* gene complement (13). The identification of the polypeptides was performed by MALDI-TOF MS. The number of peptides matching the theoretical trypsin digest of the polypeptide (Matching) is compared to the total number of detected peptides (Total). The ratio between the cumulative length of the matching peptides and the length of the protein is expressed as the percentage of peptide sequence coverage.

same insertion (GT between nucleotides 716 and 717), but for each of the four other clones (G2, G4, G9, and M2), the sequence was unique. For six of the clones (G1, G2, G8, G9, M1, and M5) the mutation would result in a C-terminal trun-

cation of 100 to 111 amino acids. For the G4 clone, a single insertion at position 778 would result in an HrcA product in which the 73 C-terminal amino acids were truncated. For the M2 clone, a substitution and a single nucleotide deletion would

TABLE 3. Effect of *M. pulmonis* MpUR1.1 growth temperature on MICs of antimicrobial peptides^a

Growth temperature	MIC (μ M) of:						
	Alamethicin	Iturin A	Surfactin	Gramicidin D	Melittin	Gramicidin S	Tetracycline
37°C	6.25	25	25	0.0015	3.12	3.12	0.1
40°C	-	-	-	$\times 8^c$	-	-	-
40°C > 37°C ^b	-	-	-	-	-	-	-

^a *M. pulmonis* MpUR1.1 was grown during nine passages either at 37°C or at 40°C. After that, these mycoplasma cultures were used as inocula to determine the MICs (μ M) of antimicrobial peptides and tetracycline at 37°C.

^b After nine passages at 40°C, *M. pulmonis* MpUR1.1 was passaged twice at 37°C, and the resulting culture was used as an inoculum to determine the MICs (μ M) of antimicrobial peptides and tetracycline at 37°C. A dash indicates that the MIC was the same as the one reported at 37°C (top row).

^c $\times 8$, the MIC was increased eightfold.

result in an even shorter HrcA truncation of only six C-terminal amino acids and a modified C terminus over the last eight residues. Interestingly, upstream from these mutations, all the *hrcA* sequences from *M. pulmonis* MpUR1.1 and the eight selected clones were identical over 700 nucleotides.

The mutations found in the G and M clones that were evaluated strongly supported that in these clones the truncated HrcA could not act as a negative regulator, and this would in turn constitutively activate the stress response in these cells. This response would possibly provide, by a yet undetermined mechanism, a certain level of resistance to melittin, gramicidin D, and tetracycline. It should also be noted that although isolates G1, G8, and M5 have identically mutated *hrcA* sequences, the three lineages have different MIC profiles for three antibiotics, suggesting that other differences must be present.

The genes which are regulated following a specific stress belong to a set that is partially overlapping a set of genes regulated during a different type of stress. With this in mind, we evaluated whether an experimentally induced heat stress response in *M. pulmonis* would be associated with decreased sensitivity towards antimicrobial peptides or other antibiotics.

Heat shock response in *M. pulmonis* and resistance to antimicrobial peptides. During a natural *M. pulmonis* infection, part of the host's response consists of an increased body temperature (i.e., fever), which represents a stress for the mycoplasma. We evaluated whether these conditions would lead to increased MICs of the antimicrobial peptides. *M. pulmonis* MpUR1.1 was passaged nine times under an incubation temperature kept at 40°C, with the hope that these conditions would mimic the temperature found in the rodent lung during an active *M. pulmonis* acute infection. After these passages at 40°C, the MICs of melittin, gramicidin D, and tetracycline were determined at 37°C; only the MIC of gramicidin D was found to be increased (eightfold increase) (Table 3). Interestingly, if the nine passages at 40°C were followed by two passages at 37°C before determination of the MIC, the MIC of gramicidin D was not found to be increased. This result clearly established that the passages at an increased temperature (40°C) resulted in changes in the cell metabolism that were associated with increased resistance to gramicidin D. However, this increased MIC was transient and returned to normal once the mycoplasma was grown at 37°C (Table 3).

The proteome of *M. pulmonis* MpUR1.1 after growing at 40°C was analyzed by 2-D gel electrophoresis. As expected, a number of differences with the proteome of the mycoplasma cultivated at 37°C were found (Fig. 3). Several proteins that

were upregulated at 40°C were identified by mass spectrometry (Fig. 3C). In the set of identified proteins, DnaK was the only polypeptide that was common to the proteins upregulated in the G and M clones (Fig. 1G and 3C). The other upregulated proteins included elongation factor TS (EF-TS), glyceraldehyde 3 phosphate dehydrogenase, thioredoxin reductase, and a protein of unknown function (Fig. 3C).

Complementation of the *hrcA* mutation in *M. pulmonis* G1 and M1 clones. To evaluate whether the mutation found in the *hrcA* gene in the G and M clones was the cause of the increased MICs of gramicidin D and melittin, complementation of the mutation was attempted. The wild-type *hrcA* gene was cloned with its upstream putative promoter sequences into the AflIII site of the pMPO5 plasmid (15), resulting in the pHRCA plasmid. The G1 and M1 clones, once transformed with the pHRCA plasmid, were amplified in the presence of tetracycline without subcloning, and the obtained transformants were named CG1 and CM1, respectively. We first verified that the MICs of melittin and gramicidin D for MpUR1.1 transformed with vector pMPO5 were identical to those for MpUR1.1 without plasmid vector. The MICs of gramicidin D for CM1 and CG1 were decreased 4- to 8-fold and 64-fold compared to the MICs for M1 and G1, respectively. The MIC of melittin for CM1 was decreased two- to fourfold compared to the MIC of M1. The MICs of melittin for CG1 and G1 were the same. These findings indicate that there was a partial restoration of the wild-type phenotype by the complementation of the G1 and M1 mutants with the plasmid-borne *hrcA* gene. The proteome of the CM1 clone was analyzed; it was found to be almost identical to that of *M. pulmonis* MpUR1.1 (Fig. 4), with the disappearance of upregulated proteins shared by all the G and M clones. This result indicated that the upregulated proteins in the M1 clone, and possibly in the other M and G clones, were under the direct or indirect control of the HrcA repressor. The only difference between the proteomes of the CM1 clone and *M. pulmonis* MpUR1.1 was the upregulation of a mixture of EF-Tu (major component in spot h) and enzyme I of phosphoenolpyruvate protein phosphotransferase (minor component in spot h) (Fig. 4C). At this time, we have no explanation for this difference.

The stress response that is regulated by HrcA provides the mycoplasma with increased resistance to both gramicidin D and melittin. Until now, the only replicative plasmids available for *M. pulmonis* were based on the tetracycline selection brought by the *tetM* gene. Therefore, it was not possible to conclude about a possible association between the increased

FIG. 3. Proteomic analysis of *M. pulmonis* MpUR1.1 after culture at 37°C (A) or 40°C (B). Proteins were extracted from the mycoplasma cells by method B (see Materials and Methods). Two-dimensional electrophoresis was performed as indicated in the legend to Fig. 1. Proteins were stained with Coomassie blue. Six upregulated spots were analyzed by LC-MS/MS and identified as DnaK (spot a), EF-Ts (spot b), glyceraldehyde 3 phosphate dehydrogenase (spot c), thioredoxin reductase (spot d), and protein of unknown function (spots e and f). (C) The corresponding gene (Mypu_no) is indicated according to the published nomenclature of the *M. pulmonis* gene complement (13). The number of peptides identified by LC-MS/MS, the cumulative length of these peptides compared to the total length of the protein, and the corresponding percentage of peptide coverage are indicated.

resistance for tetracycline in the G and M clones and the detected mutation in the *hrcA* gene.

DISCUSSION

In this study, we have shown that *M. pulmonis* can develop a resistance to melittin and gramicidin D. However, in spite of several attempts, resistant clones were only obtained for these two peptides out of seven that were evaluated. This result is, to some extent, in agreement with the previously described difficulty for bacteria to develop resistance to antimicrobial peptides (for a review see reference 17). The resistance of *M. pulmonis* to dermaseptin (a frog skin peptide) and polymyxins is in agreement with previously published data indicating that three other *Mycoplasma* species (*M. gallisepticum*, *M. genitalium*, and *M. mycoides* subsp. *mycoides* SC) are resistant to animal defense peptides and polymyxins (6).

The isolation of *M. pulmonis* clones with increased MICs to both melittin and gramicidin D but not to other antimicrobial peptides is surprising because these two molecules are structurally different although being both linear (Table 1). Melittin, a bee venom component, is a genetically encoded 26-residue peptide composed exclusively of amino acids identical to those found in proteins. In contrast, gramicidin D is a shorter pep-

tide (15 residues), is nonribosomally synthesized by *Bacillus brevis*, and displays a sequence of alternating D- and L-amino acids. Furthermore, while melittin is mainly α -helical in lipid bilayers, gramicidin D is believed to form transmembrane, left-handed antiparallel β -helices (10). The association between the increased MICs of these two peptides for the G and M clones and an increased MIC of tetracycline is of particular interest because this may represent a new mechanism of resistance to an antibiotic that is still widely used in the clinical setting (14).

Modifications observed in the expressed proteomes of resistant clones suggested that peptide resistance and stress response could be correlated in some way. An association between antibiotic resistance and stress response has already been reported in gram-negative bacteria (*Gracilicutes*) for which a response to osmotic stress was observed after nonbactericidal exposure to polymyxin B or cecropin (35, 36). In agreement with this hypothesis, in all the G and M clones, mutations were identified in the *hrcA* gene that has been shown to control stress response in many bacteria (32, 56) and proposed to play the same role in mycoplasmas (52, 56). The HrcA protein acts as a repressor of several genes encoding stress proteins, among them the chaperones DnaK and DnaJ and the

FIG. 4. Proteomic analysis of the *hrcA*-complemented *M. pulmonis* M1 clone. Proteins were extracted from the cells of *M. pulmonis* MpUR1.1 (A), clone M1 (B), and transformant CM1 (C) by method B (see Materials and Methods). Two-dimensional electrophoresis was performed as indicated in the legend to Fig. 1. Proteins were detected using silver staining. The seven upregulated proteins (Fig. 1) that were identified by comparing the proteomes of *M. pulmonis* MpUR1.1 and clone M1 are indicated (spots a to g). Spot h corresponds to a mixture of two proteins that are upregulated in the CM1 transformant compared to the proteome of *M. pulmonis* MpUR1.1. (D) The number of peptides identified by LC-MS/MS, the cumulative length of these peptides compared to the total length of the protein, and the corresponding percentage of peptide coverage are indicated.

protease Lon. The mutations in the *hrcA* gene in the G and M clones could result in constitutive activation of the stress response in cells. In agreement with this proposal, DnaK was identified among the proteins that were upregulated in the G and M clones, which supports the hypothesis that the *dnaK* gene is under the negative regulation of HrcA. Moreover, a computer search of HrcA-binding sequences (CIRCE elements) on the *M. pulmonis* genome revealed a potential CIRCE element upstream from the *dnaK* gene. Two other CIRCE elements, upstream from the *lon* and *dnaJ* genes, were predicted (Table 4). Although no definitive conclusion could be drawn for the regulation of these two genes because the

corresponding proteins were not identified by 2-D gel electrophoresis, our results strongly suggest that resistance to melittin and gramicidin D could result, at least in part, from a derepression of HrcA-regulated genes. The partial restoration of peptide sensitivity after complementation with the wild-type *hrcA* gene is in agreement with this proposal.

Drosocin, pyrrhocoricin and apidaecin, three proline-rich peptides produced by insects, act stereospecifically on the *Escherichia coli* heat shock protein DnaK but not on its human homolog (37). It is further known that pyrrhocoricin inhibits the ATPase activity of *E. coli* DnaK (30, 31). Although our observations do not prove that DnaK is also a target of melittin

TABLE 4. Localization of CIRCE elements in the *M. pulmonis* genome

Position	Strand	Regulated gene	No. of base pairs from start codon	CIRCE element ^a		
				5' stem	Loop	3' stem
255798	+	<i>dnaK</i> (Mypu_2230)	−175	TTAGCACTT	TATTCATTT	GAGTGCTAA
636450	−	<i>lon</i> (Mypu_5190)	−132	TTGGCACTC	TTTTGAATA	AAGTGCTAT
894436	+	<i>dnaJ</i> (Mypu_7330)	−20	TTAGCACTC	TAAGTTTAA	TTGTGCTAA

^a The nucleotides that differ from the CIRCE consensus sequence are underlined. The CIRCE consensus sequence is as follows: 5' stem, TTAGCACTC; loop, N9; 3' stem, GAGTGCTAA.

and gramicidin D, its upregulation in the mutants suggests that this protein could be involved directly or indirectly in the resistance mechanism. The presence of foreign hydrophobic molecules (here, melittin and gramicidin D) in the cytosol of *M. pulmonis* might indeed require increased amounts of the cytoplasmic chaperone DnaK, which is involved in cytoplasmic protein homeostasis.

The upregulation of enzymes involved in the energetic metabolism of *M. pulmonis* (phosphotransferase system enzymes involved in the uptake of sugars and pyruvate dehydrogenase which catalyzes the formation of acetyl-coenzyme A) may be necessary to cope with increased energy demand. Indeed, mycoplasmas use the proton motive force to activate secondary substrate transport systems. In fermentative bacteria, the proton motive force is generated by the ATPase F0F1 functioning as a proton pump activated by the ATP produced by substrate-level phosphorylation. Since in bacteria the cell energetic charge needs to be finely tuned, any loss of energy due, for example, to membrane permeabilization by amphipathic peptides should be compensated by an increased yield of energy.

The proteomic analysis of the G and M clones of *M. pulmonis* defined a set of proteins that were upregulated, plausibly as a consequence of the mutations in the *hrcA* gene. However, the proteins detected by 2-D gel electrophoresis are only a subset of the complete proteome and probably do not include very hydrophobic proteins and proteins with a high pI, a well-known limit of 2-D protein electrophoresis. Consequently, we cannot exclude the possibility that the expression of other proteins could be modified in the resistant clones. In fact, an analysis performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that the variable surface antigens of *M. pulmonis* varied among the analyzed clones (data not shown). The analysis of these polypeptides that are known to vary at high frequency (51) did not reveal any correlation with resistance to antimicrobial peptides. This is in agreement with a recent report indicating that larger versions of variable surface antigens provide *M. pulmonis* with a shield protecting it from complement lysis but not the action of smaller molecules, such as gramicidin D (45).

The occurrence of mutations in the same region of the *hrcA* gene among *M. pulmonis* clones that were independently obtained is striking and raises the possibility that this region of the genome could be a hot spot for mutations. Although the exploration of such a hypothesis would require much more data, it is of interest to note that no other mutation was found in the 700 nucleotides upstream from the mutated region of *hrcA*. The lack of a system of methyl-directed mismatch repair in mycoplasmas led to the hypothesis that they could be considered constitutive mutators

(46). If this were true, it would favor the development of antibiotic resistance as previously described for other bacteria (24). The genomes of mutator bacteria are characterized by a high frequency of mutations; in the case of mycoplasmas, this is supported at least partially by phylogenetic studies based on rRNA gene sequences which suggested that mycoplasmas have a high mutation rate and hence are in a state of rapid evolution (43, 53, 55). In the case of genome regions other than rRNA, there is a lack of experimental data to document a putative higher-than-normal frequency of mutation. However, it has been shown for many mycoplasma species that high-frequency point mutations at specific locations, such as microsatellites, is a strategy used by mycoplasmas to generate surface antigen diversity (40, 42). It was also found recently that reversible point mutations, located in a nonrepeated region of the chromosome, are responsible for the phase variation of the GapA cytidhesin in *M. gallisepticum* (54) and of the glucose phosphotransferase system permease (*ptsG*) in *M. mycoides* subsp. *mycoides* SC (23). In our study, the mutations in the *hrcA* gene occur in the same region of the gene where no statistically significant DNA repeats were found during a survey of the entire genome (42). It is possible that the stress due to the exposure to the antimicrobial peptides could favor the accumulation of mutations in particular regions of the genome. Recently, the stress associated with aging cells within a bacterial population was described as a factor influencing the mutation rate in *E. coli* (7). It remains to be determined whether the mutation in the *hrcA* gene is reversible, even at low frequency, as was documented for the mutation in the *ptsG* gene of *M. mycoides* subsp. *mycoides* SC, and whether such instability could be considered as an evolutionary strategy to escape peptide-mediated antimicrobial response during host-pathogen interaction.

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