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Role of *lrp* in the *Pseudomonas Aeruginosa* Small Colony Formation

Dinesh Diraviam Sriramulu

Division of Cell and Immune Biology, Helmholtz Centre for Infection Research, Braunschweig, Germany, 38124 Braunschweig, Germany

Corresponding author: Dinesh Diraviam Sriramulu, CIBIO-University of Trento, Via della Regole 101, Mattarello, (TN) 38060, Italy, Tel: +39 461 883093; Fax: +39 461 883937; E-mail: d.sriramulu@gmail.com

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Abstract

Formation of small colonies and slow growth are among common features of *Pseudomonas aeruginosa* isolated from the lung of cystic fibrosis (CF) patients. A slowgrowing CF lung isolate strain C that was complemented with the whole-genome cosmid library, created from an environmental isolate, revealed the role of leucine-responsive regulatory protein on the colony size, the growth and the proteome profile. Sequence analysis of *lrp* and its homologues in strain C revealed a mutation in the homologue PA3965 compared to the respective sequence of PAO. In addition to the regulation of enzymes involved in amino acid biosynthesis and metabolism, Lrp seemed to regulate the expression of chaperones (DnaK, GroEL and GroES) and the suppressor protein DksA.

Keywords: *Pseudomonas aeruginosa*; Leucine-responsive regulatory protein; Small colony variant; Adaptation; Cystic fibrosis

Abbreviations

CF, Cystic Fibrosis
Lrp, Leucine-responsive regulatory protein
TSB, Trypticase soy broth
TSA, Trypticase soy agar
AA's, Amino Acids

Introduction

Chronic lung infection by *Pseudomonas aeruginosa* in cystic fibrosis (CF) patients accounts for morbidity and mortality. Once colonized, *P. aeruginosa* cannot be eradicated even by the most aggressive antibiotic therapy (Hoiby, 1993). In the CF lung environment, strains of *P. aeruginosa* are known to evolve toward a common phenotype such as lack of flagella, amino acid auxotrophy, deficiency in lipopolysaccharides, changes in LPS fatty acid acylation, reduced secretion of virulence factors, mutator phenotype, small colony variants and mucoidy irrespective of their genetic background (Hancock et al., 1983; Luzar et al., 1985; Doring *et al.*, 1993; Romling et al., 1994a; Barth and Pitt, 1995; Oliver et al., 2000; Sriramulu et al., 2005b). The evolutionary pressure in the CF lung habitat is quite strong, even large chromosomal inversions occur in the *P. aeruginosa* genome to converge to a common phenotype (Kresse *et al.*, 2003). Formation of small colonies and slow growth are common among *P. aeruginosa* CF lung isolates (Wahba and Darrell, 1965, Sriramulu et al., 2005a). However their role was hardly addressed in CF research nor was their emergence explained. The *P. aeruginosa* strain C, a slow-growing strain with a frequently occurring genotype (Romling et al., 1994b) showed protein expression pattern with the down-regulation of several proteins involved in amino acid biosynthesis and metabolism (Sriramulu et al., 2005b) including among others. In this study, the genetic basis of the unique proteome pattern of *P. aeruginosa* strain C has been in-

vestigated.

Materials and methods

Bacterial Strains and Growth Conditions

P. aeruginosa strains (PAO ATCC 15692; strain C, a prototype of clone C, is a CF isolate; SG17M is a river water isolate from Germany) were grown in TSB and M9 minimal medium (6g/L Na2HPO4, 3g/L KH2PO4, 1g/L NH4Cl, 0.5g/L NaCl, 0.241g/L MgSO4 and 4g/L glucose). The M9 minimal medium was supplemented with casaminoacids (5 g/L) if necessary. The following antibiotics were used: For *E. coli*: kanamycin 50 µg/ml gentamycin 15 µg/ml, tetracycline 20 µg/ml; for *P. aeruginosa*: kanamycin 150 µg/ml, gentamicin 30 µg/ml, tetracycline 75 µg/ml.

Preparation and Characterization of Whole-cell Proteins

For protein analysis, *Pseudomonas aeruginosa* strain C was pre-cultured in trypticase soy broth (TSB) (1 % Tryptone, 0.5 % sodium chloride) overnight at 37°C with shaking. The pre-culture was used to prepare a lawn culture on trypticase soy agar (TSA) (1 % Tryptone, 0.5 % sodium chloride, 1.5 % agar) and incubated for 16 h at 37°C. All the preparative steps were carried out at 4°C unless otherwise mentioned. Cultures were scraped from the plate and washed twice with 0.01 M phosphate-buffered saline, pH 7.5 and finally centrifuged (6000 g for 10 min. at 4°C). Whole-cell proteins were extracted from *P. aeruginosa* strains and 600 µg protein was isoelectrically focussed in the first dimension across a linear pH gradient from 4 to 7 and mass separated in a 12-15 % pore-gradient SDS-PAGE in the second dimension (Gorg et al., 2000; Sriramulu et al., 2005b). The gels were stained overnight with Coomassie brilliant blue G250 (Biomol, Hamburg, Germany) as described (Neuhoff et al., 1988). Preferred spots were excised and processed for peptide mass fingerprinting or archived for the future use. Protein spots were excised and prepared for peptide mass fingerprinting as described (Shevchenko et al., 1996). Peptide mass fingerprint maps of trypsin-digested peptides generated by MALDITOF Reflex II (Bruker-Franzen-Analytik) mass-spectrometer were compared to a database of all translated ORFs from *P. aeruginosa* PAO1 (www.pseudomonas.com) using MS-Fit of Protein Prospector package (http://falcon.ludwig.ucl.ac.uk/ucsfhtml3.2/msfit.htm). Partial sequences of peptides were obtained by MS/MS using Micromass Q-Tof-2. All experiments were carried out at least twice.

Quantification of Free Amino Acids

Utilization of amino acids (aa) at certain optical density was estimated by measuring the quantity of free aa in the spent medium (TSB). Free AA's in the medium were quantitatively derivatized at the amino group, either primary or secondary, using phenylisothiocyanate in the presence of a base, di-isopropylethylamine (Applied Biosystems 420A Derivatizer). The derivatives, phenylthiocarbamyl-amino acids were subsequently analysed by high-pressure liquid chromatography (Applied Biosystems 130A Separation system) and quantified by comparison to the standard concentrations of the respective amino acids. Free intracellular AA's were extracted with water after heat lysis of washed bacteria and quantified as described above.

Molecular Methods

DNA extraction, manipulation and cloning were carried out according to standard procedures (Ausubel, 1994).

Construction of a Cosmid Library

A genome-wide cosmid library was constructed (Wenzel, 1996) for the fast-growing *P. aeruginosa* environmental strain SG17M, a clonal variant closely related to strain C (Romling et al., 1994b). SG17M showed a global proteome pattern like PAO (data not shown). *Sau*3AI partially-digested chromosomal DNA was size fractionated by preparative sucrose gradient ultracentrifugation and cloned into the *Bam*HI site of pLAFR3 (Staskawicz et al., 1987). The ligated-DNA was packaged into phage λ-particles *in vitro* by using the Gigapack III XL packaging extract (Stratagene). *E. coli* XL1 Blue MR was transfected with the λ-particles containing pLAFR3 cosmid DNA. After selection for tetracycline resistance, around 25,000 clones were recovered, pooled and stored at –80°C.

Tri-parental Mating

The complementation of *P. aeruginosa* C using SG17M cosmid library was carried out through tri-parental mating. The conjugation mixture consisting of the recipient (*P. aeruginosa* strain C), the donor strain (which carries cosmid library) and the helper pRK2013 (which carries *tra* gene for transfer) at the ratio 1:5:5 was spotted on to LB agar plate and incubated at 37°C for 8 h. Transconjugants, which

formed large-sized colonies were selected after plating the diluted conjugation mixture on LB agar plate containing tetracycline and gentamicin.

Cosmid Mutagenesis

Complementing cosmid was mutated *in vitro* using the EZ::TN™ <KAN-2> insertion kit (Epicentre) and reintroduced into strain C. Clones showing the wildtype colony morphology (small colonies) were selected, cosmids isolated and sequenced using primers provided by the manufacturer to identify the candidate gene.

Cloning

The genes *lrp* (from PAO) and PA3965 (from PAO and C) were amplified by PCR using primers lrp(F)HindIII (5'–GGAAAGCTTTGTTTTTCCTGCTTTTGGAC–3'); lrp(R)*Eco*RI (5'– CCT GAATT CGACGGTGTTTCCCG TCTATG–3') and PA3965(F)HindIII (5'–GGAA AGCTTT TGCGC AATAAA TCACTCAAA–3'); PA3965(R)*Eco*RI (5'–CCTGAATTCAACAGGCTGCCGATTATACG–3') respectively. The amplicons were purified using the Qiaquick PCR purification kit (Qiagen) and double-digested with *Hind*III and *Eco*RI. Double-digested fragments were separately cloned in to pBBR1MCS-2 (Kovach et al., 1995) between *Hind*III and *Eco*RI restriction sites and mobilized into strain C through tri-parental mating. Transconjugants were selected against kanamycin and gentamicin.

Results

Whole-cell Proteome *P. Aeruginosa* C

P. aeruginosa strain C grew slower in rich medium (TSB) in the log phase than PAO (Fig. 1). Two-dimensional separation of whole-cell proteins revealed characteristic protein expression profile for the slow-growing CF-lung isolate C (Fig. 2a). The proteome of strain C cytosolic fraction separated with pH range 4-7 showed a huge number of protein spots with lower intensity as compared to PAO (Sriramulu et al., 2005b). Most notably, such a unique proteome pattern was not only shown by strain C (Fig. 2a) isolated during the onset of the colonization process but also by a few other CF isolates, which belong to clone C, from mid- and late- phases of chronic lung infection (data not shown). Proteins belonging to different functional categories were identified with the help of the available whole genome sequence database of PAO (www.pseudomonas.com). Previous study (Sriramulu et al., 2005b) showed that proteins involved in biosynthesis as well as degradation of several amino acids were down regulated in the strain C proteome (Fig 2a).

Increased Utilization of Free Amino Acids by *P. Aeruginosa* Strain C

Growth pattern as observed in minimal medium supplemented with casaminoacids showed that strain C could reach an optical density as that of PAO (Fig. 1b and data not shown). The cystic fibrosis lung environment was reported to be rich in amino acids (Barth and Pitt, 1996). As several enzymes involved in aa metabolism were down regulated in the strain C proteome, it was reasoned that the strain C might adapt well to the amino acid-rich CF-lung (Barth and Pitt, 1996) environment by the utilization of AA's from the medium. The amount of unutilised AA's from the spent medium (TSB) was measured after incubation of bacteria up to stationary phase. Although most of the AA's were already used the intake of proline, tyrosine, isoleucine and leucine was significantly higher by strain C ($P < 0.05$) as compared to PAO (Fig. 3a and data not shown). To unravel the fate of

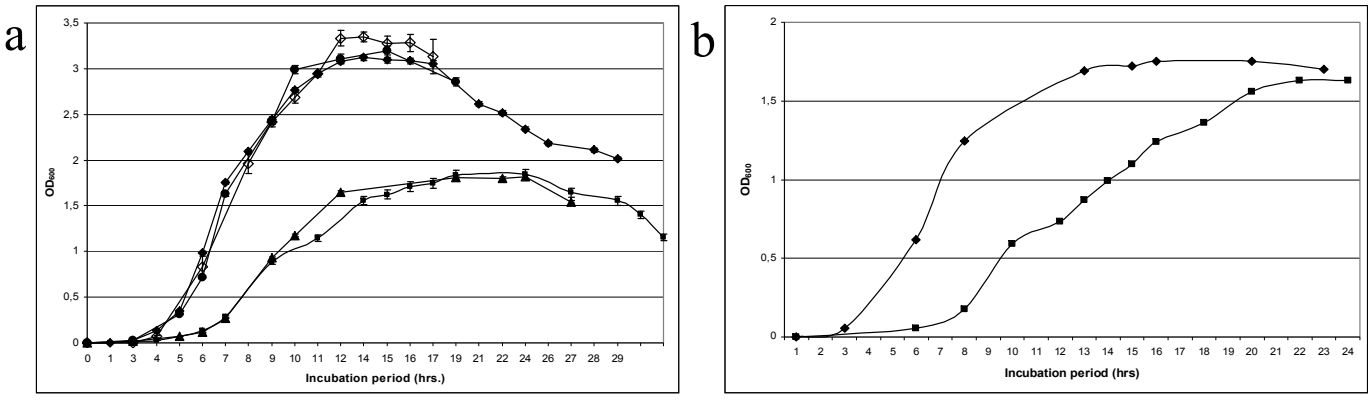

Figure 1: Growth pattern of *P. aeruginosa* PAO (♦), strain C (■), SG17M (◊), strain C - vector control (▲) and strain C complemented with lrp (•) (a) in the rich medium (TSB) and (b) in the M9 minimal medium supplemented with amino acids.

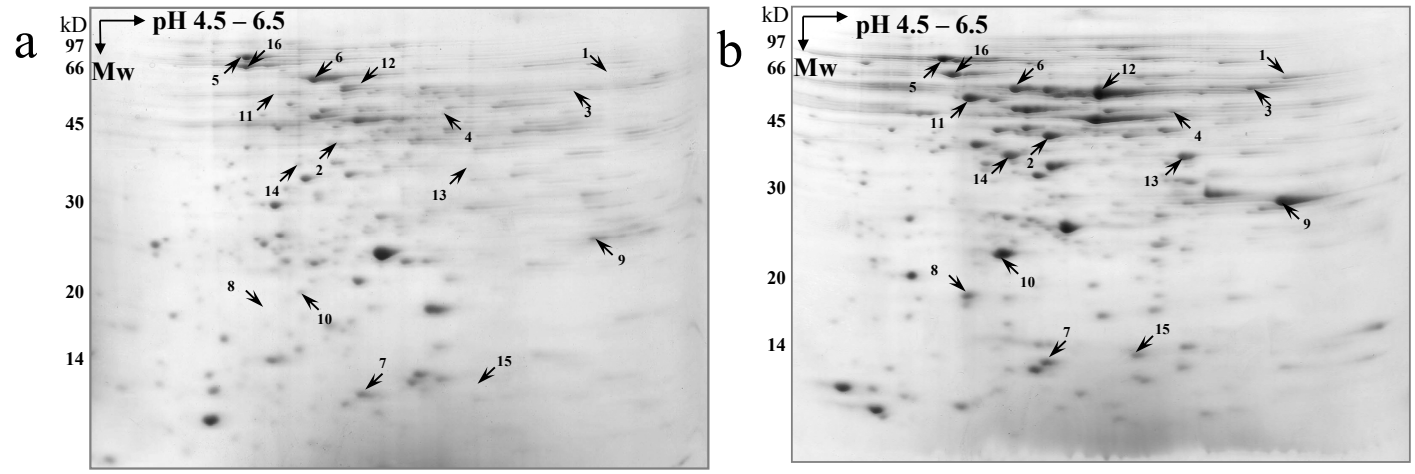

Figure 2: Whole-cell proteome of *P. aeruginosa* strain C (a) vector control and (b) complemented with *lrp* contained in a cosmid. Numbered arrows indicate the identity of protein spots listed in Table I.

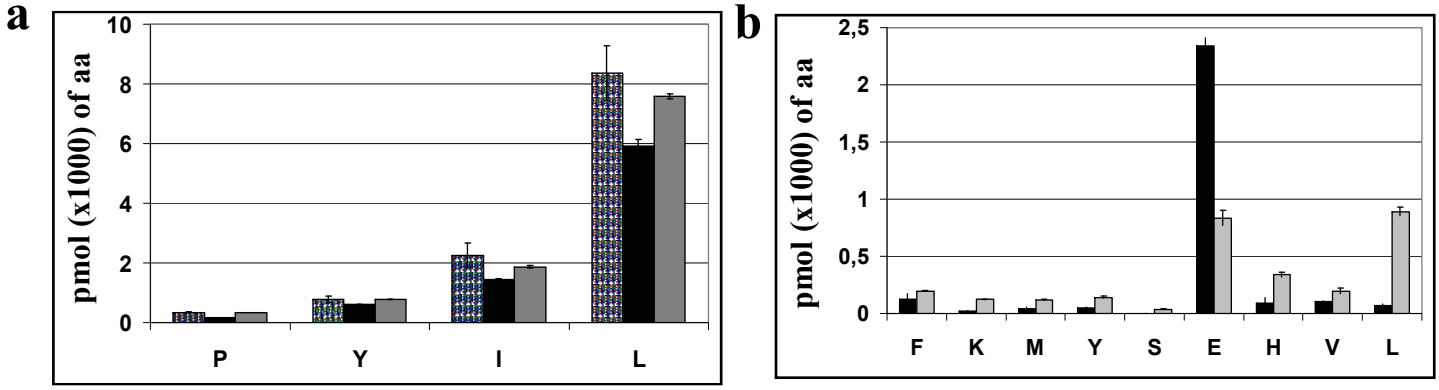

Figure 3: (a) Utilization of free amino acids in TSB (dotted bar) by *P. aeruginosa* PAO (black bar) and strain C (grey bar). (b) Intracellular concentrations of free amino acids in *P. aeruginosa* PAO (black bar) and strain C (grey bar).

aa, intracellular concentrations were measured in *P. aeruginosa* PAO and C. Histidine, valine, leucine, phenylalanine, lysine, methionine, tyrosine and serine were found in significantly higher amounts ($P < 0.05$) in the intracellular environment of the strain C (Fig. 3b). Whereas, the intracellular concentration of glutamate was higher in *P. aeruginosa* PAO compared to that of strain C.

Complementation of *P. Aeruginosa* Strain C

To identify the gene responsible for the unique behaviour of *P. aeruginosa* C, a whole-genome cosmid library of the environmental isolate *P. aeruginosa* SG17M, closely related to strain C and which could grow as PAO was used for complementation. A single cosmid with an insert size of about 35 kb was found to increase the strain C colony size. The cosmid did also restore growth in liquid medium (Fig. 1a). To identify the candidate gene responsible for the characteristics of C, a negative-selection method through *in vitro* transposon mutagenesis on the cosmid was adopted. The cosmid clone, which did not restore growth, was selected. The insertion point of the transposon was located in the open reading frame (ORF) designated PA5308, which was homologous to the *lrp* gene in *E. coli*.

Role of Lrp in *P. Aeruginosa* CF-specific Phenotype

In addition to *lrp*, there are two other *lrp*-homologues (PA2082 and PA3965) as indicated by the PAO genome (www.pseudomonas.com). Lrp from *P. aeruginosa* PAO is 79% similar to its counterpart in *E. coli*, whereas, homologous proteins encoded by ORFs PA2082 and PA3965 showed 57% and 63% similarity respectively. Both of the *lrp*-homologues from the strain C were sequenced and there was no mutation in the PA2082 gene. However, the PA3965 gene from the strain C had a mismatch (F79L) mutation in

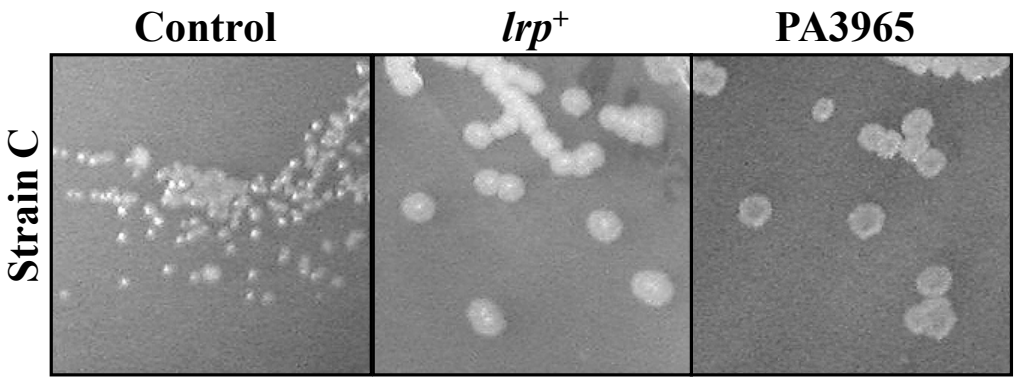

Figure 4: Colony morphology of *P. aeruginosa* strain C complemented with *lrp* or its homologue PA3965 (from PAO).

Spot no.	Identification	Gene	MW (KDa)	pI
1	Lipoamide dehydrogenase-Val	PA2250	48.6	6.16
2	4-hydroxyphenylpyruvate dioxygenase	PA0865	39.9	5.10
3	Branched-chain alpha-keto acid dehydrogenase	PA2249	45.8	5.84
4	2-oxoisovalerate dehydrogenase-Alpha subunit	PA2247	45.3	5.67
5	DnaK protein	PA4761	68.4	4.79
6	GroEL protein	PA4385	57.1	5.04
7	GroES protein	PA4386	10.3	5.20
8	Suppressor protein DksA	PA4723	17.3	5.04
9	Alkyl hydroperoxide reductase subunit C	PA0139	20.5	5.89
10	Superoxide dismutase	PA4366	23.7	6,63
11	Trigger factor	PA1800	48.6	4.83
12	ATP synthase Alpha chain	PA5556	55.4	5.34
13	Succinyl-CoA synthetase Alpha chain	PA1589	30.3	5.79
14	Electron transfer flavoprotein Alpha-subunit	PA2951	31.4	4.98
15	Azurin precursor	PA4922	16.0	8.14
16	Ribosomal protein S1	PA3162	61.9	4.83

Table I: Proteins up-regulated in the proteome of *P. aeruginosa* strain C complemented with *lrp*

addition to six mis-sense mutations. Next step was to study the direct effect of *lrp* and PA3965 genes, which were cloned and mobilized in to the strain C. Transconjugants carrying an intact *lrp* or PA3965 formed larger colonies and grew faster than the wildtype (Fig. 4 & 1a).

Additional Proteins Regulated by Lrp

From the Fig.2b, it was evident that a number of proteins (Table I) involved in amino acid biosynthesis and metabolism were upregulated in the lrp-complemented strain C. In addition, novel proteins, which were not reported previously to be under the control of Lrp are controlled by that regulatory protein. Chaperones like DnaK, GroEL and GroES and the mutation suppressor DksA were upregulated when the strain C was complemented with *lrp*.

Discussion

In this study, the proteome of the *P. aeruginosa* clone with a representative genotype found in hospitals throughout Europe (Romling *et al.*, 2005) was analysed. Particularly, *P. aeruginosa* strain C, which was isolated during the onset of the colonization process in the CF-lung was chosen.

The proteome map of *P. aeruginosa* strain C was completely different from that of the reference strain PAO as evident from our previous study (Sriramulu et al., 2005b). In particular, variations in the expression of enzymes involved in amino acid metabolism could be observed. Several enzymes involved in amino acid biosynthesis were down regulated in strain C in addition to individual enzymes involved in different metabolic pathways, which indicated that the strain C is dependent to some extent on the external source of amino acids. Almost all 20 amino acids were found at higher concentrations in the CF airways and proposed to play a significant role in the emergence and maintenance of *P. aeruginosa* auxotrophic for the biosynthesis of amino acids during the course of chronic colonization (Barth and Pitt, 1996, Thomas et al., 2000; Barth and Pitt, 1995). Differ-

ential expression of proteins involved in amino acid metabolism had been reported in *P. aeruginosa* during the course of biofilm formation (Sauer et al., 2002). The presence of amino acids in the artificial sputum medium was shown to have a pronounced effect on the proteome pattern of *P. aeruginosa* PAO (Sriramulu et al., 2005a). The *P. aeruginosa* strain C could not grow in the ASM without amino acids (data not shown), which indicated the adaptation of strain C to the amino acid-rich CF lung environment. Based on the global changes in amino acid metabolism in *P. aeruginosa* strain C, speculation towards the inactivation of a major regulatory factor was considered.

Lrp is a global regulatory protein that activates or represses genes that encode biosynthetic and catabolic enzymes in *E. coli* (Newman and Lin, 1995). The expression of Lrp is highly repressed in the presence of amino acids in addition to its autogenous regulation (Newman and Lin, 1995; Bukau, 1993). As amino acid-rich medium like TSB was used to grow bacteria it was most likely that the expression of Lrp by *P. aeruginosa* might have been repressed especially by leucine. But the presence of an Lrp-homologue (PA3965) might have had a compensatory role in the case of *P. aeruginosa* PAO grown in TSB. Whereas, down regulation of several proteins involved in amino acid metabolism in strain C proteome could be explained by the mutation found in the Lrp-homologue PA3965. Provision of an extrachromosomal copy of either *lrp* or its homologue PA3965 to *P. aeruginosa* strain C not only ameliorated the expression of proteins involved in amino acid metabolism but also resulted in big-sized colonies and faster growth compared to the wildtype. CF-lung environment was reported to be rich in amino acids that promoted the growth of auxotrophic *P. aeruginosa* (Barth and Pitt, 1996). Therefore, the repression of Lrp and in turn the repression of proteins involved in the amino acid metabolism in *P. aeruginosa* is obvious in the CF lung environment. The global role of Lrp has recently been shown to be involved in the modulation of virulence in uropathogenic *E. coli* and *Salmonella enterica* serovar Typhimurium (Baek et al., 2009, Muller et al., 2009).

The whole-cell protein analysis of *P. aeruginosa* strains revealed additional proteins (GroEL, GroES, DnaK and DksA), which seem to be regulated by the Lrp protein. Hsp60, encoded by the *groEL* gene and Hsp70, encoded by the *dnaK* gene are the two major chaperones involved in the heat-shock response (Bukau, 1993). Up-regulation of GroEL, GroES and DnaK proteins by strain C in response to Lrp is an addition to the Lrp-regulon. DksA is known to play a role in virulence of *Salmonella enterica* serovar Typhimurium (Turner et al., 1998) and *Shigella flexneri* (Mogull et al., 2001). DksA was shown to positively regulate quorum-sensing-dependent virulence genes in *P. aeruginosa* (Jude et al., 2003). In this study, the positive regulation of DksA by Lrp is evident from its up-regulation by *P. aeruginosa* strain C in the presence of Lrp. Another study reinforced the global role of Lrp by reconstructing its regulatory network in *E. coli* (Cho et al., 2008). Taken together, Lrp protein seems to play a global role in the nutrient-based adaptation of *P. aeruginosa* to the CF lung environment. Though there is a limited functional conservation of Lrp among related bacteria (Lintner et al., 2008), a detailed genome-wide study in *P. aeruginosa* clonal-variants might expand the size of the regulon due to potential regulation of genes present in genomic islands.

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