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RND-4 efflux transporter gene deletion in *Burkholderia cenocepacia* J2315: a proteomic analysis

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Abstract

**Background:** The *Burkholderia cenocepacia* J2315 is Gram-negative bacterium that is a pathogen for cystic fibrosis (CF) patients. It displays a high-level of resistance to most antimicrobial drugs. In Gram-negative bacteria, the Resistance-Nodulation-Cell Division (RND) transporter family has a poorly understood role in multidrug resistance. In a previous publication we analysed the RND-4 and RND-9 transporters by microarray analysis. The obtained results suggested that only RND-4 contributes to the antibiotic resistance. The aim of this study was to investigate the role of this efflux transporter from a proteomic point of view.

**Methods:** We quantitatively compared the intracellular proteome of the deletion mutant *B. cenocepacia* J2315 impaired in RND-4 transporter (BCAL2820-22) with that of the wild type strain using two-dimensional electrophoresis.

**Results:** The results pointed out 70 differentially expressed proteins, of which 49 were identified by mass spectrometry. We found that in RND-4 mutant strain, 13 protein spots were up-regulated whilst 35 were down-regulated. One spot was detected only in wild type J2315. Fifty percent of the 35 down-regulated proteins belong to the following functional categories: “amino acids transport and metabolism”, “nucleotides transport and metabolism”, “lipid transport and metabolism”, “translation”, “ribosomal structure and biogenesis”. Conversely, forty-six percent of 13 the up-regulated proteins belong to the following functional categories: “energy production and conversion”, “post-translational modification”, “protein turnover, chaperones”.

**Conclusions:** These results indicate that in *B. cenocepacia* J2315 the RND-4 gene deletion affects, directly or indirectly, some traits of cell physiology, suggesting for this transporter a wider role than just in drug resistance.

**Keywords:** *Burkholderia cenocepacia* J2315, cystic fibrosis, RND efflux pumps, two-dimensional electrophoresis, mass spectrometry

Introduction

The Burkholderia cepacia complex (Bcc) comprises about 17 related opportunistic pathogens that are able to infect the respiratory tract of cystic fibrosis (CF) patients and chronic granulomatous disease patients [1]. It has also been reported as a cause of bacteraemia [2]. Up to 8% of patients are colonized by Bcc for months or even years. Several Bcc species are transmissible strains as they can spread from one CF patient to another [3]. These bacteria are also able to multiply and multiply in disinfectants [4]. Bcc bacteria are difficult to eradicate since they appear to be intrinsically resistant to many antibiotics [5], so therapy is often aimed at decreasing bacterial load during exacerbations. This is the main reason of Bcc emergence as an important cause of morbidity and mortality in CF patients. Multi-drug resistance (MDR) in CF isolates is defined as resistance to the agents belonging to at least two of three classes of antibiotics; aminoglycosides, quinolones and β-lactam agents (monobactams and carbapenems) [6]. *In vivo*, under antimicrobial pressure, *B. cenocepacia* can achieve resistance to essentially all classes of antimicrobial drugs. Among the mechanisms of resistance, enzymatic inactivation as well as alteration of drug target and cell wall permeability, has been reported [7]. Another aspect related to drug resistance is the presence in the of *B. cenocepacia* genome of genes encoding all five major families of efflux systems [8]. In Bcc these efflux systems are involved in resistance to chloramphenicol, trimethoprim and fluoroquinolones. The Resistance-Nodulation-cell Division (RND) family plays an important role among mediators of multi-drug resistance in Gram-negative bacteria.

In *B. cenocepacia* J2315 sixteen operons of RND efflux transporters were identified [8,9]. The one named ceoB cluster (BCAM2549–2552, BCAM2554) is responsible for resistance to chloramphenicol, trimethoprim, and ciprofloxacin [10], while two other RND systems (BCAL1675 and BCAM1947) were shown to be up-regulated during...
growth in CF sputum [11]. Interestingly, two *B. cenocepacia* RND pumps (BCAL1674–BCAL1676 or RND-3, and BCAL2822–BCAL2820 or RND-4) have been shown to contribute significantly to the multi drug resistance [12] because of the involvement in secretion of the quorum-sensing signal AHL. Recently, we studied two previously characterized RND efflux systems: RND-4 (BCAL2820–22) and RND-9 (BCAM1945-1947). By an in silico analyses these two systems have been shown to belong to the HAE-1 family which comprises proteins responsible for antibiotics extrusion [13]. We constructed *B. cenocepacia* strains inactivated in these operons thus achieving single mutants D4 and D9, as well as a double mutant D4-D9 [12]. Microarray experiments were performed and confirmed by qRT-PCR, while phenotype characterization as well as Phenotype MicroArray analysis were conducted. The results revealed that RND-4 contributes to the antibiotic resistance in *B. cenocepacia*, whilst RND-9 was only marginally involved. Moreover the RND-4 displayed additional phenotypic traits such as the up-regulation of some genes related to both flagellum and chemotaxis, that are important for the pathogenesis. The RND-4 mutant showed also an enhanced swimming mobility and biofilm formation with respect to the wild type strain [14].

Even though transcriptomic analysis is a sensitive and global tool to catalog genome-wide information, proteomics reports also the final state of proteins which undergo post-translational modifications. Therefore, proteomic analysis has become an additional choice to search for proteins at post-transcriptional levels. However, detection sensitivity of this approach is relatively low, and insoluble proteins can be lost during preparation. In the last few years, proteomic analysis of *Burkholderia pseudomallei*, *Burkholderia thailandensis*, *B. cepacia*, and *B. cenocepacia* has provided a proteomic reference map to study factors important for virulence, stress tolerance, and viability [15-17]. The present study deals with the proteomic characterization of the deletion mutant of *B. cenocepacia* J2315 impaired in RND-4 efflux pump. We tried to assess the RND-4 role in the cell physiology of *B. cenocepacia* J2315 by comparing the intracellular proteome of *B. cenocepacia* D4 mutant with that of the wild type strain using two-dimensional electrophoresis analysis coupled to mass spectrometry.

**Materials and Methods**

**Materials**

All chemicals were purchased from Sigma Aldrich (Italy), unless mentioned otherwise.

**Bacterial strains and growth conditions**

The bacterial strains used in this work were the wild type strain *B. cenocepacia* J2315 and the mutant D4 harbouring a deletion of the two ORF BCAL2822–BCAL2820 (RND-4) [12]. These strains were grown in Luria-Bertani (LB) medium by shaking up to an OD<sub>550</sub> = 0.5. Ten ml of each culture were then harvested by centrifugation (3500g for 15 min at 4°C). Cells were gently washed three times with 50 mM TrisHCl, pH 7.5 to remove components of the culture media. The pellet was recovered and stored at −80°C until use. Three independent growth experiments (biological replicates) were performed and analysed for both wild type and D4 mutant strains.

**Cell lysis and protein extraction**

The pellet was resuspended in the lysis buffer containing 100 mM Tris-HCl pH 9, 1% SDS, 1 mM DNase (Roche), 1 mM RNase (Roche) supplemented with protease inhibitor (Protease Inhibitor Cocktail®, Roche). Cell lysis was performed by sonication and the cell debris was removed by centrifugation (8,000g; 1 h; 4°C). Protein were precipitated by adding five volumes of cold (−20°C) acetone. After 2h at −20°C precipitates were recovered by centrifugation at 12,000g for 30 min at 4°C, supernatants were discarded, and pellets were washed once with cold acetone, and vacuum-dried for 5 min. Protein pellets were resolved in a buffer containing 8 M urea, 4% 3-cholamidopropyl dimethylammonium-1-propane sulfonate (CHAPS), 65 mM dithioerythritol (DTE). Protein concentration was determined by the standard Bradford method (Bio-Rad).

**Two-dimensional electrophoresis (2-DE)**

The protein samples obtained were separated by two-dimensional gel electrophoresis as previously described [18,19]. Briefly, isoelectric focusing (IEF) was carried out on linear wide-range immobilized pH gradients (IPGs; pH 4.0–7.0; 18-cm-long IPG strips; GE Healthcare, Uppsala, Sweden) and achieved using an Ettan IPGphor™ system (GE Healthcare). Protein sample (800 µg) was loaded by cup loading in the Ettan IPGphor Cup Loadind Manifold™ (GE Healthcare) after the rehydration of the IPG strips with 350 µl of rehydration solution (8 M urea, 2% (w/v) CHAPS, 0.5% (w/v) DTE) supplemented with 0.5% (v/v) carrier ampholyte and a trace of bromophenol blue, overnight at room temperature. The strips were focused at 16°C according to the following electrical conditions: 200 V for 1 h, 300 V for 1 h, from 300 to 3,500 V in 30 min, 3,500 V for 4h, 5,000 for 2h, from 5,000 to 8,000 V in 30 min, and 8,000 V until a total of 100,000 V/ h was reached, with a limiting current of 50 µA/strip. After focusing, strips were equilibrated in 6 M urea, 2% (w/v) SDS, 2% (w/v) DTE, 30% (v/v) glycerol and 0.05 M Tris-HCL pH 6.8 for 12 min and, subsequently, for 5 min in the same urea/SDS/Tris-HCl buffer solution where DTE was substituted with 2.5% iodoacetamide (IA). The equilibrated strips were placed on top of 9−16% polyacrylamide linear gradient gels (18 cm × 20 cm × 1.5 mm) and embedded in 0.5% heated low-melting agarose in SDS electrophoresis running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3). The methylenebisacrilamide was the cross-linker used in the 9-16% gradient. SDS-PAGE was performed in a PROTEAN
II x1 cell gel electrophoresis unit (Bio-Rad) at 10°C and at 40mA/gel constant current, until the dye front reached the bottom of the gel, according to Hochstrasser et al., [20]. Gels were stained with Colloidal Coomassie Blue G-250 [21].

**Image analysis and statistics**

Two gel replicates (technical replicates) for each biological replicate (three independent growth experiments) were performed, so that, for each bacteria strain (wild type and D4), 6 gels were analysed. Stained 2-DE images were digitized using the Epson expression 1680 PRO scanner. Image analysis, including alignments and matching between spots, was carried out using ImageMaster 2D Platinum software version 7.0 (GE Healthcare). The protein expression profiles of *B. cenocepacia J2315* wild type and RND-4 mutant were compared. The protein spot volumes were normalized automatically against the total spot volume of the gel using the software. Relative spot volume (%V) \( V_{\text{single spot}} / V_{\text{total spots}} \) (where V is the integration of the optical density over the spot area) was used for quantitative analysis in order to decrease experimental errors. The %V of each protein spot on the 6 replicate 2-DE gels was average and the standard deviation (SD) was calculated for wild type J2315 and mutant D4 strains. In order to select the appropriate statistical test, the normal distribution of variable was tested using GraphPad Prism version 4.0. Then, a two-tailed non-paired Student’s t-test was performed to determine if the relative change in %V was statistically significant between the two *B. cenocepacia* strains \( p \leq 0.05 \). To these spots we also applied an arbitrary fold change cut-off of \( \geq 1.5 \). For each spot the relative change in %V of D4 versus J2315 (indicated with "fold change D4/J2315") was calculated by dividing the average from the D4 gels by the average from the J2315 gels. Only the protein spots with a p-value \( \leq 0.05 \) and a threshold of \( \geq 1.5 \)-fold change were selected for mass spectrometry analysis.

**Protein identification by Mass Spectrometry (MS)**

Electrophoretic spots, visualized by Colloidal Coomassie staining protocol, were manually excised, destained, and acetonitrile dehydrated. Forty ml of 10 mM DTT in 20 mM ammonium bicarbonate were added to each excised spot and incubated at 56°C for 45 minutes. DTT solution was removed and 40 ml of 100 mM IA solution in 20 mM ammonium bicarbonate were added and incubated at room temperature for 30 minutes in the dark. IA solution was removed and spots were dehydrated for 10 minutes with 80% acetonitrile (ACN) and dried 10 minutes in speedvac. A trypsin solution (0.25mg/ml) in 50 mM ammonium bicarbonate was added for in-gel protein digestion by overnight incubation at 37°C. Solutions containing digested peptides were recovered and 20 ml of 1% TFA 50% ACN were added to each spot and sonicated for 10 minutes to maximize peptide recovery. At the end, all recovered peptide solutions were combined and concentrated for each spot separately.

Proteolytic peptides were mixed with CHCA matrix solution (5 mg/ml alpha-cyano-4-hydroxycinnamic acid (CHCA) in 0.1% TFA/70% ACN, v/v) in a 1:1 ratio, and 2 ml of this mixture was spotted onto the MALDI target. Spots were analyzed using a 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada), equipped with a laser emitting at \( \lambda = 355 \) nm with a repetition rate of 200 Hz. The mass spectrometer was controlled by the 4000 Series Explorer, version 3.5.2 program. For MS analyses, about 2000 spectra were acquired for each spot in the reflector positive mode in the mass range of 650 to 4000 m/z, with 50 ppm mass tolerance (external calibration). MS/MS data acquisition was performed on all spots by automatic selection of the best 20 precursor ions. MS/MS acquisitions were then carried out using air as collision gas at a pressure of \( \sim 3.0 \times 10^{-5} \) torr and collision energy of 1 kV. Approximately 2000 spectra were added up for each spot. The peaks were de-isotoped and only those with s/n >5 were retained for interpretation. MS/MS data were pre-processed by the computer program GPS (Global Proteomics Server Explorer) version 3.6 (Applied Biosystems).

Identification of proteins was established using MASCOT search engine version 2.1 (Matrix Science, Boston, MA) with the last updated FASTA version of EST genomic database from NCBI GenBank (downloaded from ftp blast databases from NCBI as EST-others) and created using translated information for *Burkholderia cepacia* complex (Bcc) and all other *Burkholderia* members annotated. Methionine oxidation, Asn and Gln deamidation, and acetylation were selected as variable modifications in the search. The tolerance for precursor ion and MS/MS fragment mass values was set at 50 ppm and 0.3 Da, respectively and a peptide charge state of +1. Trypsin digestion and two possible missed cleavages were used. Only the 2 top-ranked peptide matches were taken into consideration for protein identification. Probability-based Molecular Weight Search scores were estimated by comparison of the search results against the estimated random match population, and were reported as -10log10(p) where p is the absolute probability. Individual MS/MS ions scores \( >25 \) indicated identity or extensive homology \( (p \leq 0.05) \) for the MS/MS ion search. Protein scores greater than 69 are significant \( (p < 0.05) \). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

Functional annotation was performed using Clusters of Orthologous Groups of proteins (COG) classification of NCBI database (http://www.ncbi.nlm.nih.gov/COG/).

**Bioinformatic functional analysis**

To identified statistically over-represented (enriched) Gene Ontology terms among the differentially expressed proteins identified by MS analysis, we used David Bioinformatics Resource (version 6.7) (Database for Annotation, Visualization and Integrated Discovery; http://david.abcc.ncifcrf.gov/)

**Figure 1.** Representative Colloidal Coomassie-stained 2-D gel electrophoresis (pH 4–7) of the intracellular proteins of *B. cenocepacia* J2315 wild type strain (a) and of RND-4 deletion mutant (b). Whole-intacellular proteins (800 µg) were separated on IPG strips with a pH gradient of 4–7, followed by separation on 9–16% linear gradient SDS-PAGE. The differentially expressed protein spots between wild type and RND-4 mutant strain, are displayed with black circles. The spots labeled with black circles and numbers were identified by MALDI-TOF/TOF mass spectrometry. They are listed in Table 1 and in Supplement Table.

*B. cenocepacia* J2315, the protein expression pattern of the wild type strain were compared with that of the RND-4 deletion mutant strain (D4) by proteomic approach. First, total intracellular proteins purified from wild type J2315 and D4 mutant strains were resolved by two-dimensional electrophoresis using non-linear pH 3-10 IPG strips and we found that the most were present in the pH 4-7 range (data not shown). Therefore, we proved to resolve cellular proteins on pH 4-7 IPG strips and we found that this pH range allows a better separation of proteins as demonstrated by other authors [15-17,24]. To achieve statistically significant results, for each strain, three independent growth experiments were performed (biological replicates). For each biological replicate, two 2-DE gels (technical replicates) were obtained. Hence, for each strain a total of 6 gels were computer-aided analysed and approximately 1200 spots were resolved. The replicate gels showed a very similar protein pattern indicating a high degree of reproducibility of the experimental procedures. The quantitative image analysis was performed comparing the relative spot volume (%V) of protein spots detected in the D4 gels to those of spots detected in the wild type J2315 gels. A two-tailed non-paired Student’s *t*-test was performed to determine if the relative change in %V was statistically significant between the two *B. cenocepacia* strains (*p*-value ≤0.05). Among the protein spots statistically significant, we selected the spots with a fold change greater than 1.5. The fold change in protein expression was calculated for each spot as the ratio of normalized %V between D4 and wild type J2315. The comparative proteomic analyses revealed 70 differentially expressed protein spots with a *p*-values ≤0.05 and a fold change ≥1.5. Among these protein spots, 18 were up-regulated whereas 51 were down-regulated in D4 mutant strain. One protein spot was detected only in wild type 2-DE gels. These spots are marked with black circles in Figure 1, panel a and b.

Protein identification by MALDI-TOF/TOF mass spectrometry

The 70 differentially expressed proteins were subjected to MALDI-TOF/TOF mass spectrometry analysis. The identification of 49 proteins was successfully performed by the combined mass finger-printing and MS/MS sequencing, using MASCOT search engine version 2.1 with the last updated FASTA version of EST genomic database from NCBI GenBank and created using translated information for Bcc and all other *Burkholderia* members annotated. The remaining 21 differentially expressed protein spots could not be identified because either they were present in too low amount in 2-DE gels or because their mass spectra had not matched with any of the proteins reported in the databases. Among the 49 identified protein spots, in D4 mutant strain respect to the wild type J2315 strain, 13 were up-regulated whereas 35 were down-regulated. One spot (spot n. 31) was detected only in the wild type strain. These identified spots are marked with black circles and numbers in Figure 1.

[22,23]. All the differentially expressed proteins identified by MS were included in the analysis. Fisher’s exact test was used to check for significant over-representation (*p*<0.05) of GO terms in the submitted dataset against the *B. cenocepacia* genome.

**Results**

Comparative analysis of *B. cenocepacia* J2315 and RND-4 deletion mutant proteomes

In order to establish the cellular processes affected by the RND-4 multidrug efflux pump gene deletion in
(panels a, b). The quantitative 2-DE analysis and the identity of these 49 protein spots, corresponding to 48 different proteins, are summarized in Table 1 and in the Supplement Table. The experimental isoelectric point (pI) and molecular weight (Mw) values of most of the identified proteins, detected by 2-DE analysis, were similar to those theoretically predicted from the genome sequence (Supplement Table). Only for one spot (spot n. 26) clear change in migration was observed. This spot displayed significant differences in the determined molecular weight with a shift toward higher mass. This discrepancy might be due to a post-translational modification of the corresponding native protein. Forty-eight protein spots were identified as annotated proteins of B. cenocepacia J2315. For one protein spot (spot n. 21), with good quality spectra, no significant match to any sequence in the current B. cenocepacia J2315 annotated proteome was obtained. This protein spot was identified as an annotated protein from another Bcc strain, that is B. ambifaria MC40-6.

Chromosome localization of the genes encoding the identified proteins
We analyzed the distribution of the genes encoding the 48 identified proteins affected, directly or indirectly, by RND-4 deletion, on the B. cenocepacia J2315 genome (Table 1 and Figure 2). The genome is divided into 4 circular replicons: chromosome 1 (3.87 Mbp), chromosome 2 (3.217 Mbp), chromosome 3 (0.876 Mbp) and one plasmid (92.7 bp). Previous studies on this genome revealed that chromosome 1 contains most of genes involved in central metabolism whereas chromosomes 2 and 3 contain a greater proportion of genes encoding accessory functions [8]. Interestingly, genes encoding the 48 identified proteins in this study are unequally distributed between the three chromosomes. In detail, the 89.6% of these genes are distributed on chromosome 1, the 8.3% on chromosome 2 and the 2.1% on chromosome 3 (Figure 2). Moreover no protein encoded by the plasmid genes was found among these differentially expressed proteins.

Functional classification of identified proteins
The identified proteins were clustered into functional categories according to Clusters of Orthologous Groups of proteins (COG) (http://www.ncbi.nlm.nih.gov/COG/). In this way, 16 different COG categories were identified (Table 1 and Figure 3). “Amino acid metabolism”, “Nucleotide metabolism”, “Lipid metabolism”, “Translational, ribosomal structure and biogenesis” are the functional categories that include the highest number of proteins with a lower content in D4 mutant. Conversely, “Energy production” and “Post-translational modification” are the functional categories that include the highest number of proteins with a higher content in D4 mutant. For two proteins (spots n. 48 and 49), no functional COG category could be annotated. Further details on the 16 functional categories are given below.

COG E category: amino acid transport and metabolism
Four different proteins, associated with amino acid biosynthesis process, exhibited a lower content in D4 mutant, including pyrroline-5-carboxylate reductase (spot n. 2), 2-isopropylmalate synthase (spot n. 3), histidinol dehydrogenase (spot n. 4) and succinyldiaminopimelate transaminase (spot n. 5) (Table 1 and Figure 3). Conversely, the oligopeptidase A abundance was higher in the mutant respect to the wild type strain (spot n. 1).

COG F category: nucleotide transport and metabolism
The levels of five proteins implicated in purine and pyrimidine biosynthesis (Table 1 and Figure 3) were decreased in D4 strain compared to wild type J2315. Specifically, we identified one protein involved in pyrimidine biosynthesis like orotidine 5’-phosphate decarboxylase, pyrF (spot n. 6) and 4 proteins involved in purine biosynthesis such as phosphoribosylformylglycinamidine synthase, purl (spot n. 7), phosphoribosylamine–glycine ligase, purB (spot n. 8), phosphoribosylaminomidazole-succinocarboxamidase synthase, purC (spot n. 9). Moreover, we found down-regulated the enzyme guanylate kinase, gmk (spot n.10) implicated in the biosynthesis of guanosine 5’-triphosphate (GTP) and dGTP. Gmk also functions in the recycling of 3’5’-cyclic diguanylic acid (c-di-GMP). As concerns this enzyme, it is reported to play a regulator role in the prokaryotic biofilm lifestyle and recent evidence also links this molecule to virulence [25].

COG I category: lipid transport and metabolism
Four proteins related to this category were down-regulated in mutant D4 (Table 1 and Figure 3). In particular, we identified succinyl-CoA:3-ketoacid-coenzyme A transferase subunit B (spots n. 11) involved in the synthesis and degradation of ketone bodies; poly-beta-hydroxybutyrate polymerase (spots n. 12) involved in butanoate metabolism and potentially in rhamnolipid synthesis. The rhamnolipid synthesis is one of the three virulence-associated-pathways in B. cenocepacia J2315 [26]. Although no report demonstrates the synthesis of rhamnolipids in B. cenocepacia, Dubey et al., emphasized the presence in Burkholderia thailandensis of genes orthologous to those responsible for the synthesis of rhamnolipids in P. aeruginosa [26,27]. We also identified a putative oxidoreductase (spot n. 13) and the 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase, lspG (spot n. 14) involved in biosynthesis of isoprenoids. This protein is a key enzyme of the mevalonate-independent pathway, essential in Bcc bacteria, for the biosynthesis of a terpenoid precursor. Isoprenoids are ubiquitous in bacteria as membrane components, quinones in electron transport and pigments [28,29].
Table 1. Mass Spectrometry identification of differentially expressed proteins in RND-4 mutant strain respect to the wild type B. cenocepacia J2315 strain.

<table>
<thead>
<tr>
<th>COG Functional Category and Protein Name</th>
<th>Spot No</th>
<th>Locus tag</th>
<th>Accession number</th>
<th>COG</th>
<th>Chr Loc</th>
<th>Fold change (D4 / J2315)**</th>
<th>p-value</th>
<th>Score</th>
<th>Sequence coverage (%)</th>
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<tr>
<td><strong>Amino acid transport and metabolism (E)</strong></td>
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<td></td>
<td></td>
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<tr>
<td>oligopeptidase A</td>
<td>1</td>
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<td>gi</td>
<td>206560575</td>
<td>0339E</td>
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<td>1.5</td>
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<td>pyrroline-5-carboxylate reductase</td>
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<td>gi</td>
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<td>0345E</td>
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<td>YP_002232820.1/</td>
<td>gi</td>
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Table 1 continuation.

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**Function unknown (S)**

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**Hypothetical proteins**

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* Functional annotation and protein name according to the COGs functional classification of NCBI database (http://www.ncbi.nlm.nih.gov/COG/).
†Spot numbers refer to identified spots marked with circles and numbers in the representative 2-DE gels of Figure 1 (panel a and b).
‡Locus tag was obtained from NCBI database.
§NCBI RefSeq and NCBI ID.
||Numbers refer to COGs assignments derived from NCBI database.
¶Localization of corresponding genes on chromosome 1, chromosome 2, or chromosome 3 of B. cenocepacia J2315.
**Relative change in %V of D4 protein spot versus J2315 protein spot calculated by dividing the Avg. from the D4 gels by the Avg. from the J2315 gels.
††A two-tailed Student’s t-test was performed to determine if the relative change in %V was statistically significant between the two B. cenocepacia strains. (p≤0.05 was considered statistically significant).
‡‡MASCOT score (Matrix Science, London, UK; http://www.matrixscience.com). Protein scores greater than 69 are significant (p<0.05).
§§Sequence coverage = (number of the identified residues/total number of amino acid residues in the protein sequence) x 100%.

**COG G category: carbohydrate transport and metabolism**

We identified the enzyme phosphoenolpyruvate synthase (spot n. 15), involved in pyruvate metabolism, that was up-regulated in mutant D4.

**COG Q category: secondary metabolites biosynthesis, transport and catabolism**

One protein belonging to this COG category, exhibited a lower content in D4 mutant (spot n. 16). It was identified as putative fumarylacetocetate (FAA) hydrolase family protein also known as ureidoglycolate lyase. It is involved in the tyrosine catabolic pathway.

**COG P category: inorganic ion transport and metabolism**

Three identified proteins belong to the inorganic ion transport and metabolism. Two of them, the putative cation transporter efflux protein (spots n. 18) and the putative ferritin DPS-family DNA binding protein (spot n. 19) were down-regulated in mutant D4, while the spot 17, identified as peroxidase/catalase KatB, resulted up-regulated. DPS-family proteins and KatB play a key role in protecting B. cenocepacia from oxidative stress mediated, for example, by organic hydroperoxides [30].

**COG H category: coenzyme transport and metabolism**

One protein, belonging to the coenzyme transport and metabolism, resulted down-regulated in D4 mutant strain (spot n. 20). It was identified as putative methyltransferase family protein (UbiE).

**COG C category: energy production and conversion**

Three proteins involved in energy production and conversion exhibit a higher content in D4 mutant. These include a protein of the TCA cycle named tartrate/fumarate...
subfamily Fe-S type hydro-lyase subunit alpha (spot n. 21),
NADH dehydrogenase subunit C (spot n. 22) involved in
oxidative phosphorylation and the putative phenylacetic
acid degradation oxidoreductase (spot n. 23).

COG M category: cell wall/membrane/envelope
biogenesis
Among the down-expressed proteins in D4 mutant, we
identified the enzyme UDP-N-acetylmuramoylalanine-
D-glutamate ligase (MurD) (spots n. 24) that is involved
in peptidoglycan biosynthesis. MurD catalyzes the
addition of glutamate to the nucleotide precursor UDP-
N-acetylmuramoyl-L-alanine during cell wall formation.
Recently MurD has been computationally identified as
one of the essential genes in B. cenocepacia J2315 that
can be candidate drug targets [26].

COG O category: posttranslational modification,
protein turnover, chaperones
Three proteins related to this COG functional category
resulted up-regulated in D4 mutant. Specifically, we
identified family S9 serine peptidase (spot n. 25), chaperonin
GroEL (spot n. 27) and co-chaperonin GroES (spot n. 28). We
also identified two protein spots down-regulated as thiol
peroxidase (spot n. 29) and chaperonin GroEL (spot n. 26).
As concerns GroEL and GroES they are the major chaperone
system in all bacteria and are important for folding and
function of proteins as well as for stress responses [31-33].
The decrease in content of a GroEL protein spot (spot n. 26),
with a different molecular weight respect to the native
protein (spot 27), was interesting. This variation in molecular
mass suggests a post-translational modifications (PTM). Post-
translational modifications are known to play a major role
in eukaryotes but less is known about their role in bacterial
physiology. Further investigations are needed.

COG TK category: signal transduction mechanisms
and transcription
Two proteins are involved in signal transduction mechanisms
and transcription. One protein, (spot n. 30) identified as
osmolarity response regulator, was down-regulated in D4
mutant. The other protein spot (spot n. 31), identified as
tetracycline repressor protein (TetR) was not detected in
D4 mutant 2-DE gels. It could be due to a non-expression
of the corresponding gene or to a reduced expression level,
lower than the sensitivity of the technique. The resistance of
Gram-negative bacteria against tetracyclines is frequently
triggered by drug recognition of the Tet repressor. This
enables expression of the resistance protein TetA, which is
responsible for active efflux of tetracycline [34]. The down-
regulation of this gene is in agreement with the well-
established antimicrobial susceptibility of the B. cenocepacia
D4 mutant [12].

COG D category: cell cycle control, cell division,
chromosome partitioning
As concerns this COG category we identified the cell division
protein FtsA (spot n. 32) which was up-regulated in D4;
the septum formation inhibitor (MinC) (spot n. 33), which
displayed a lower content in mutant D4. This protein, in
Escherichia coli, acts an inhibitor of division, blocking the
formation of polar Z-ring septa [35].

COG L category: replication, recombination and repair
We identified in D4 an up-expression of the recombinase A
(RecA) (spot n. 34), involved in homologous recombination,
DNA repair and the induction of the SOS response.
Conversely, the excinuclease ABC subunit B (uvrB) (spot n.
35), a component of UvrABC repair system that catalyzes
the recognition and processing of DNA lesions, resulted
down-regulated.

COG J category: translation, ribosomal structure and
biogenesis
Five proteins involved in this functional category, exhibited
a lower content in mutant, including phenylalanyl-tRNA
synthetase subunit alpha (spots n. 36); ribonuclease PH
(spots 38) that is involved in maturation of tRNA precursors;
elongation factor P (spot n. 39) involved in peptide bond
synthesis; 16S rRNA-processing protein RimM (spot n. 40)
and GTP-dependent nucleic acid-binding protein EngD (spot
n. 41) involved in translation-associated GTPase. Only one
protein spot, identified as elongation factor G (spot n.37)
was up-regulated. This protein promotes GTP-dependent
translocation of the ribosome during translation. These
data suggest that the deletion of the RND-4 efflux pump
negatively affects protein synthesis, by inhibiting some proteins involved in translation. Recently, a possible correlation between translation and antibiotic resistance of pathogen bacteria was showed in a proteomic study by A. Madeira et al., [36]. This hypothesis is consistent with the identification in Pseudomonas aeruginosa of some translation and transcription genes involved in aminoglycoside resistance [37].

COG R category: general function prediction
Four proteins belonging to this functional category resulted down-regulated in D4 mutant. Specially, we identified putative short-chain dehydrogenase (spot n. 42), putative decarboxylase (spot n.43), metallo-beta-lactamase superfamily protein (spot n. 44) and putative hydrolase protein (spots n. 45). B. cenocepacia J2315 is resistant to β-lactam antibiotics. This resistance appears to be caused by several mechanisms, including the induction of chromosomal β-lactamases [8]. There are at least four β-lactamases encoded in the J2315 genome, (BCAM0393, BCAM1179, BCAM2165 and BCAS0156) and several β-lactamase family proteins with β-lactamase domains [38]. Moreover, these proteins are known to play a role in RND efflux pumps and resistance to antibiotics. In fact, we have demonstrated that the RND-4 mutant is more susceptible than the wild type J2315 strain when exposed to a variety of drugs including β-lactams such as aztreonam [12].

COG S category: function unknown
Two identified proteins are involved in this COG category. They were identified as LysM domain/BON superfamily protein (spots n. 46) and hypothetical protein BCAL2897 (spot n. 47). These proteins were down-regulated in D4 mutant. As concerns the first protein, the Lysin domain is a protein component present in a variety of enzymes involved in bacterial cell wall degradation. This domain may have a general peptidoglycan binding function. Several proteins with this domain, such as staphylococcal IgG binding proteins and Escherichia coli intimin, are involved in bacterial pathogenesis [39].

Enrichment of gene functional annotations
In order to enhance the biological interpretations of our differentially expressed protein list we performed, by the web-accessible program DAVID (version 6.7), a gene-GO term enrichment analysis. As a result, a series of statistically over-represented Biological process (BP) and Molecular Function (MF) GO terms, was obtained (p<0.05). Results of the Fisher’s enrichment analysis are displayed in Figure 4. We identified a remarkable enrichment of BP terms relating to nucleobase, nucleoside and nucleotide metabolic process (GO:0055086), nucleotide metabolic process (GO:0009177), nucleoside phosphate metabolic process (GO:0006753) and purine nucleotide metabolic process (GO:0006163). Most of the top score MF terms included ontologies related to purine ribonucleotide binding (GO:0032555),
Figure 4. Enriched GO terms for Biological Process (BP) and Molecular Function (MF) through David software. Fisher’s exact test was used to check for significant over-representation (p<0.05) of GO terms in the differentially expressed protein list against the *Burkholderia cenocepacia* genome.
B. cenocepacia J2315 deficient in RND-4 efflux pump gene. RND-4 belongs to the Resistance-Nodulation-Cell Division family, whose members catalyze the active efflux of many antibiotics and chemotherapeutic agents [40]. Several studies suggest that RND efflux systems play important roles in bacterial pathogenesis, participating in colonization and persistence of bacteria in the host [41,42]. Previous data revealed that RND-4 efflux pump is involved in the extrusion of different toxic compounds, including some antibiotics. Moreover, in our recent paper, transcriptomic and phenomic analyses suggested that the deletion of RND-4 coding genes might have a “pleiotropic” effect in the B. cenocepacia J2315 metabolism [14]. These findings were in part confirmed by the proteomic analysis performed in this study. The comparisons of proteomic data with previously published microarray data was carried out. Only three proteins identified in this work as differentially expressed in RND-4 mutant respect to the wild type, correspond to differentially expressed genes deriving from the microarray analysis (BCAM1744, BCAL3258 and BCAL1952) (data not shown). The lack of (perfect) correspondence between proteomic and microarray analyses might be due to changes at translational and/or post-translational level [43]. In this proteomic analysis, we demonstrated that the RND-4 gene deletion affects, directly or indirectly, the cellular quantity of a complex series of proteins with known and unknown function revealing the occurrence of adaptive changes. In particular, the amount of some proteins involved in amino acid transport and metabolism, translation and nucleotide synthesis are lower in D4 mutant suggesting a decreased protein and DNA synthesis in this mutant strain. In agreement with these data, the bioinformatic functional analysis, revealed a statistically enrichment of biological process and molecular function GO terms associated with nucleotide metabolism. Moreover the level of four proteins involved in lipid synthesis and of one protein involved in peptidoglycan biosynthesis are also down-regulated. These data may reflect an alteration in plasma membrane permeability and cell wall composition. We also found down-expressed or undetected proteins with a putative role in antibiotic resistance. Specially, we identified the tetracycline repressor protein, tetR (BCAL3258) and the metallo-β-lactamase superfamily protein (BCAL1818). These findings are in agreement with the well-established role of the RND efflux pumps protein family in resistance to antibiotics as reported in our previous paper [12]. Among the up-regulated proteins in mutant D4, we identified proteins involved in post-translational modification, protein turnover or chaperones. The higher content of molecular chaperones, like GroEL and GroES, whose function is to maintain appropriate protein folding, is consistent with the presence of environmental stresses in mutant strain. Although an alteration of a protein amount does not per se necessary mean the alteration of the corresponding metabolic pathway, and much of the hypotheses raised remain conjectural these results may constitute the basis of further mechanistic studies.

Conclusion
The proteomic analysis, in agreement with the previous transcriptomic study, suggests the idea that the effect of the RND-4 deletion is not “narrowed” to the extrusion of toxic compounds. The RND-4 gene deletion affects the cell metabolism leading to a variation in some proteins associated with the well-established intracellular virulence determinants (amino acid metabolism, lipid biosynthesis, protection from oxidative stress) [44]. This could be the consequence of a stress condition connected to the loss of the RND-4 protein or it could be linked to the physiological role of this protein efflux pump. In future we will perform more detailed study to clarify the possible role of the RND-4 deletion in the induction of a direct or indirect stress condition. We are completely aware that the scenario concerning the role that RND-4 (and other efflux pumps) coding genes in vivo is still unclear and that the metabolic networks in which they are involved are to be clarified. However, in this context this proteomic analysis sheds a first light on the cellular processes that could be affected by RND-4 deletion in B. cenocepacia J2315. We are currently extending our proteomic analyses on extracellular and membrane proteins of the D4 mutant. The comparison of these proteomic patterns to those of the wild type strain will be instrumental in characterizing the general and specific functions of RND-4 for B. cenocepacia J2315 biology and pathogenesis.

Additional files

<Supplement Table S1>

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
Conceived and designed the experiments: TG, SR and AM. Analyzed the data: TG, SR, FM and RF. Wrote the paper: TG, SR and RF. MCP, S Bazzini, S Buroni, CU and EP helped to draft the manuscript. All authors read and approved the final manuscript.
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