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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 survive 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

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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 qRTPCR, 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_{550} = 0.5. Ten ml of each culture were then harvested by centrifugation (3500*g* 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 (8,000*g*; 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 12,000*g* 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, isoeletric 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 xi 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) ($V_{single\ spot}/V_{total\ spots}$, 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 ≥ 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 ≤0.05 and a threshold of ≥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 λ= 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 ~3.0×10^6 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/)

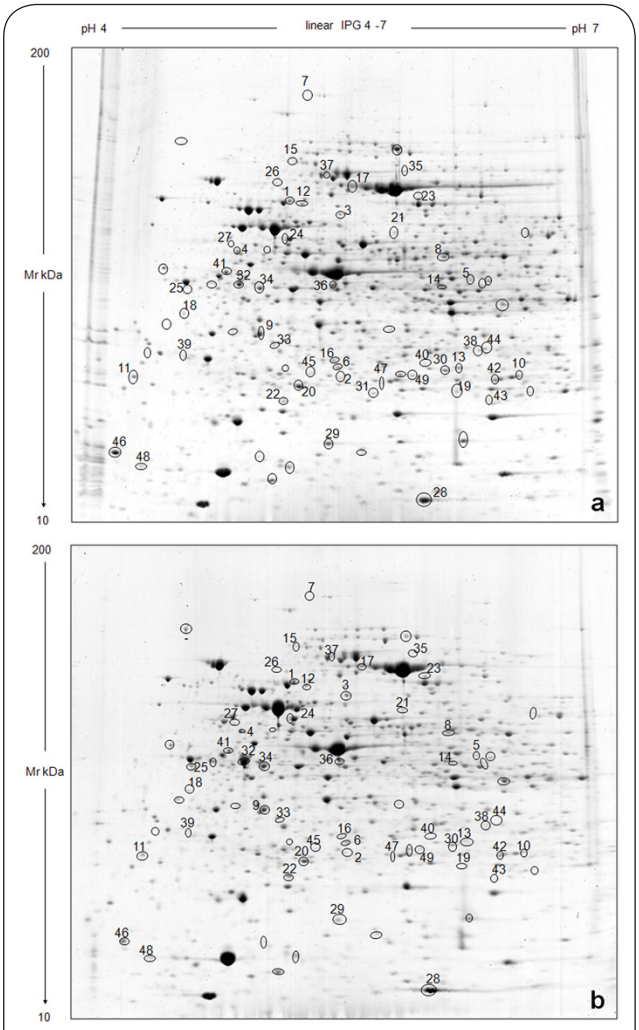

Figure 1. Representative Colloidal Coomassie-stained 2-D gel electrophoresis (pH 4–7) of the intracellular proteins of *B. cenocepacia* J2315 wild type strain (**a**) and of RND-4 deletion mutant (**b**). Whole-intacellular proteins (800 µg) were separated on IPG strips with a pH gradient of 4–7, followed 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**.

[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 *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**

(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, purD (spot n. 8), phosphoribosylaminoimidazole-succinocarboxamide 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*, Dubeau *et al.,* emphasized the presence in *Burkholderia thailandesis* 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, IspG (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 precusor. 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.

COG Functional Category and Protein Name *	Spot No †	Locus tag ‡	Accession number §	COG ‖	Chr Loc ¶	Fold change (D4 / J2315)**	p-value ††	Score ‡‡	Sequence coverage (%) §§
Amino acid transport and metabolism (E)									
oligopeptidase A	1	BCAL2213	YP_002231340.1/ gi|206560575	0339E	1	1.5	0.001	147	34%
pyrroline-5-carboxylate r eductase	2	BCAL3292	YP_002232396.1/ gi|206561631	0345E	1	-5.6	0.015	68	14%
2-isopropylmalate synthase	3	BCAM0187	YP_002232820.1/ gi|206562057	0119E	2	-2.5	0.029	310	38%
histidinol dehydrogenase	4	BCAL0312	YP_002229478.1/ gi|206558718	0141E	1	-1.7	0.005	247	28%
succinyldiaminopimelate transaminase	5	BCAL2100	YP_002231226.1/ gi|206560462	0436E	1	-2.6	0.011	67	3%
Nucleotide transport and metabolism (F)									
orotidine 5'-phosphate decarboxylase	6	BCAL3400	YP_002232502.1/ gi|206561737	0284F	1	-1.6	0.008	600	67%
phosphoribosylformylglycinami-dine synthase	7	BCAL1987	YP_002231114.1/ gi|206560350	0046F	1	-2.5	0.037	239	25%
phosphoribosylamine-glycine ligase	8	BCAL2389	YP_002231513.1/ gi|206560748	0151F	1	-1.8	0.022	356	39%
phosphoribosylaminoimidazole-succinocarboxamide synthase	9	BCAL2838	YP_002231940.1/ gi|206561175	0152F	1	-1.7	0.004	161	77%
guanylate kinase	10	BCAL3012	YP_002232122.1/ gi|206561357	0194F	1	-2.0	0.028	116	22%
Lipid transport and metabolism (I)									
succinyl-CoA:3-ketoacid-coenzyme A transferase subunit B	11	BCAL1473	YP_002230604.1/ gi|206559840	2057I	1	-1.6	0.033	309	37%
poly-beta-hydroxybutyrate polymerase	12	BCAL1863	YP_002230990.1/ gi|206560226	3243I	1	-1,5	0.006	530	36%
putative oxidoreductase	13	BCAL0194	YP_002229360.1/ gi|206558600	2084I	1	-1.7	0.005	239	71%
4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	14	BCAL1884	YP_002231011.1/ gi|206560247	0821I	1	-2.3	0.025	74	36%
Carbohydrate transport and metabolism (G)									
phosphoenolpyruvate synthase	15	BCAL2074	YP_002231200.1/ gi|206560436	0574G	1	1.8	0.003	369	25%
Secondary metabolites biosynthe-sis, transport and catabolism (Q)									
putative FAA-hydrolase family protein	16	BCAM2707	YP_002235308.1/ giI206564545	0179Q	2	-2.1	0.012	86	7%
Inorganic ion transport and metabolism (P)									
peroxidase/catalase KatB	17	BCAL3299	YP_002232403.1/ gi|206561638	0376P	1	2.5	0.020	206	25%
putative cation transporter efflux protein	18	BCAL0907	YP_002230061.1/ gi|206559300	4535P	1	-1.5	0.008	482	49%
putative ferritin DPS-family DNA binding protein	19	BCAL3297	YP_002232401.1/ gi|206561636	0783P	1	-15	0.002	81	35%
putative methyltransferase family protein	20	BCAS0206	YP_002153597.1/ gi|197295056	2226H	3	-1.5	0.001	67	54%

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Table 1 continuation.

COG Functional Category and Protein Name *	Spot No †	Locus tag ‡	Accession number §	COG ‖	Chr Loc ¶	Fold change (D4 / J2315)**	p-value ††	Score ‡‡	Sequence coverage (%) §§
Energy production and conversion (C)									
tartrate/fumarate subfamily Fe-S type hydro-lyase subunit alpha (*B. ambifaria* MC40-6)	21	BamMC406_2112	YP_001808807.1/ gi|172061155	1951C	1	1.6	0.011	66	9%
NADH dehydrogenase subunit C	22	BCAL2342	YP_002231466.1/ gi|206560701	0852C	1	1.9	0.023	283	32%
putative phenylacetic acid degradation oxidoreductase	23	BCAL0408	YP_002229574.1/ gi|206558814	1012C	1	2.5	0.044	117	32%
Cell wall/membrane/envelope biogenesis (M)									
UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase	24	BCAL3464	YP_002232566.1/ gi|206561801	0771M	1	-1.9	0.038	547	34%
Posttranslational modification, protein turnover, chaperones (O)									
family S9 serine peptidase	25	BCAM1744	YP_002234356.1/ gi|206563593	1404O	2	4.3	0.027	106	21%
chaperonin GroEL (HSP60 family)	26	BCAL3146	YP_002232255.1/ gi|206561490	0459O	1	-2.8	0.044	468	58%
chaperonin GroEL (HSP60 family)	27	BCAL3146	YP_002232255.1/ gi|206561490	0459O	1	2.8	0.009	68	3%
co-chaperonin GroES	28	BCAL3147	YP_002232256.1/ gi|206561491	0234O	1	1.5	0.016	246	79%
thiol peroxidase	29	BCAL3424	YP_002232526.1/ gi|206561761	2077O	1	-1.6	0.011	713	76%
Signal transduction mechanisms; Transcription (TK)									
osmolarity response regulator	30	BCAL2011	YP_002231138.1/ gi|206560374	0745TK	1	-2.6	0.009	133	36%
tetracycline repressor protein	31	BCAL3258	YP_002232363.1/ gi|206561598	1309K	1	- ‖‖	- ‖‖	187	50%
Cell cycle control, cell division, chromosome partitioning (D)									
Cell division protein FtsA	32	BCAL3458	YP_002232560.1/ gi|206561795	0849D	1	3.6	0.009	330	48%
septum formation inhibitor	33	BCAL3027	YP_002232137.1/ gi|206561372	0850D	1	-1.8	0.024	256	55%
Replication, recombination and repair (L)									
recombinase A	34	BCAL0953	YP_002230107.1/ gi|206559346	0468L	1	2.3	0.008	158	44%
excinuclease ABC subunit B	35	BCAL2302	YP_002231427.1/ gi|206560662	0556L	1	-2.9	0.016	171	30%
Translation, ribosomal structure and biogenesis (J)									
phenylalanyl-tRNA synthetase subunit alpha	36	BCAL1485	YP_002230615.1/ gi|206559851	0016J	1	-2.2	0.011	689	52%
elongation factor G	37	BCAL0231	YP_002229398.1/ gi|206558638	0480J	1	3.2	0.041	169	55%
ribonuclease PH	38	BCAL3014	YP_002232124.1/ gi|206561359	0689J	1	-3.5	0.001	76	22%
elongation factor P	39	BCAL2858	YP_002231958.1/ gi|206561193	0231J	1	-2.3	0.016	137	31%
16S rRNA-processing protein RimM	40	BCAL2927	YP_002232027.1/ gi|206561262	0806J	1	-2.4	0.036	69	23%
GTP-dependent nucleic acid-binding protein EngD	41	BCAL0387	YP_002229553.1/ gi|206558793	0012J	1	-1.8	0.018	498	50%

Table 1 continuation.

COG Functional Category and Protein Name *	Spot No †	Locus tag ‡	Accession number §	COG ‖	Chr Loc ¶	Fold change (D4 / J2315)**	p-value ††	Score ‡‡	Sequence coverage (%) §§
General function prediction only (R)									
putative short-chain dehydrogenase	42	BCAL3198	YP_002232307.1/ gi|206561542	4221R	1	-1.7	0.030	92	36%
putative decarboxylase	43	BCAL2368	YP_002231492.1/ gi|206560727	1611R	1	-2.9	0.016	176	41%
metallo-beta-lactamase superfamily protein	44	BCAL1818	YP_002230945.1/ gi|206560181	0491R	1	-2.2	0.021	118	31%
putative hydrolase protein	45	BCAL0916	YP_002230070.1/ gi|206559309	0637R	1	-1.8	0.027	59	7%
Function unknown (S)									
LysM domain/BON superfamily protein	46	BCAL1952	YP_002231080.1/ gi|206560316	1652S	1	-2.2	0.001	127	46%
hypothetical protein BCAL2897	47	BCAL2897	YP_002231997.1/ gi|206561232	1556S	1	-1.6	0.011	211	63%
Hypothetical proteins									
hypothetical protein BCAL1645	48	BCAL1645	YP_002230773.1/ gi|206560009	None	1	1.6	0.004	157	26%
hypothetical protein BCAM0600	49	BCAM0600	YP_002233224.1/ gi|206562461	None	2	-1.5	0.017	489	75%

* 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. ($p \leq 0.05$ 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%.
‖‖This protein spot was detected only in the 2-DE gels of B. cenocepacia wild type strain.

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 fumarylacetoacetate (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

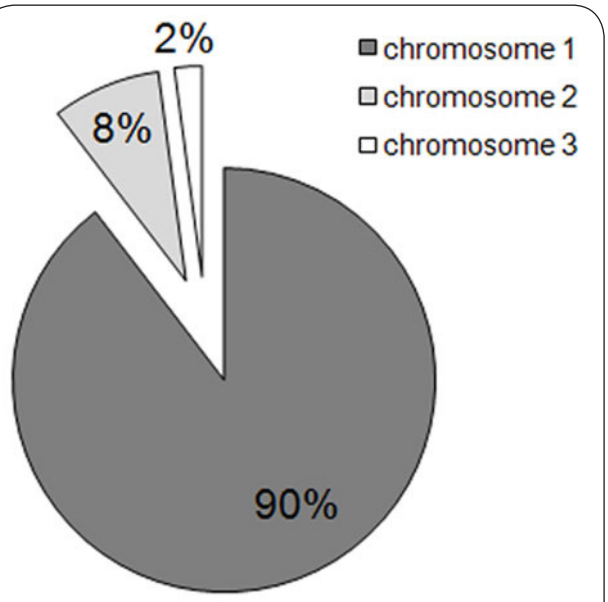

Figure 2. Distribution of genes encoding the differentially expressed proteins, identified by MS, on *B. cenocepacia* J2315 chromosomes.

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 (spot n. 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

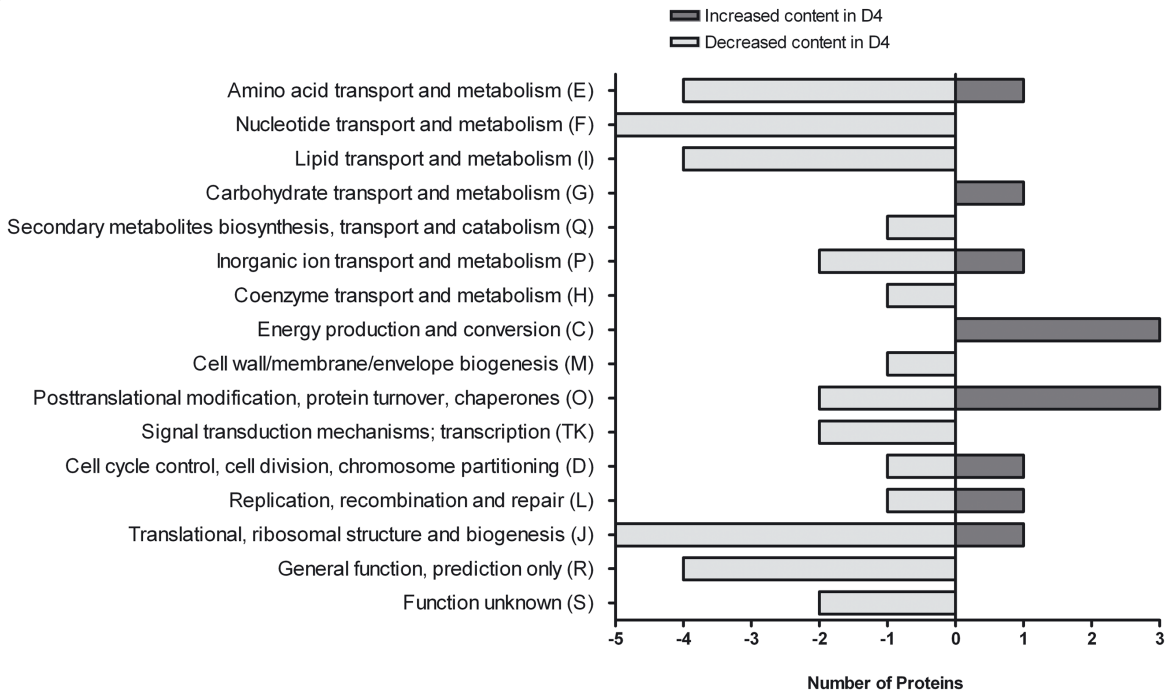

Figure 3. Number of proteins whose content is higher or lower in RND-4 mutant strain compared to wild type J2315 strain, clustered by COG functional annotation of NCBI (http://www.ncbi.nlm.nih.gov/COG/). COG assignments for individual proteins are listed in **Table 1**.

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 *Pseudomons 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:0009117), 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),

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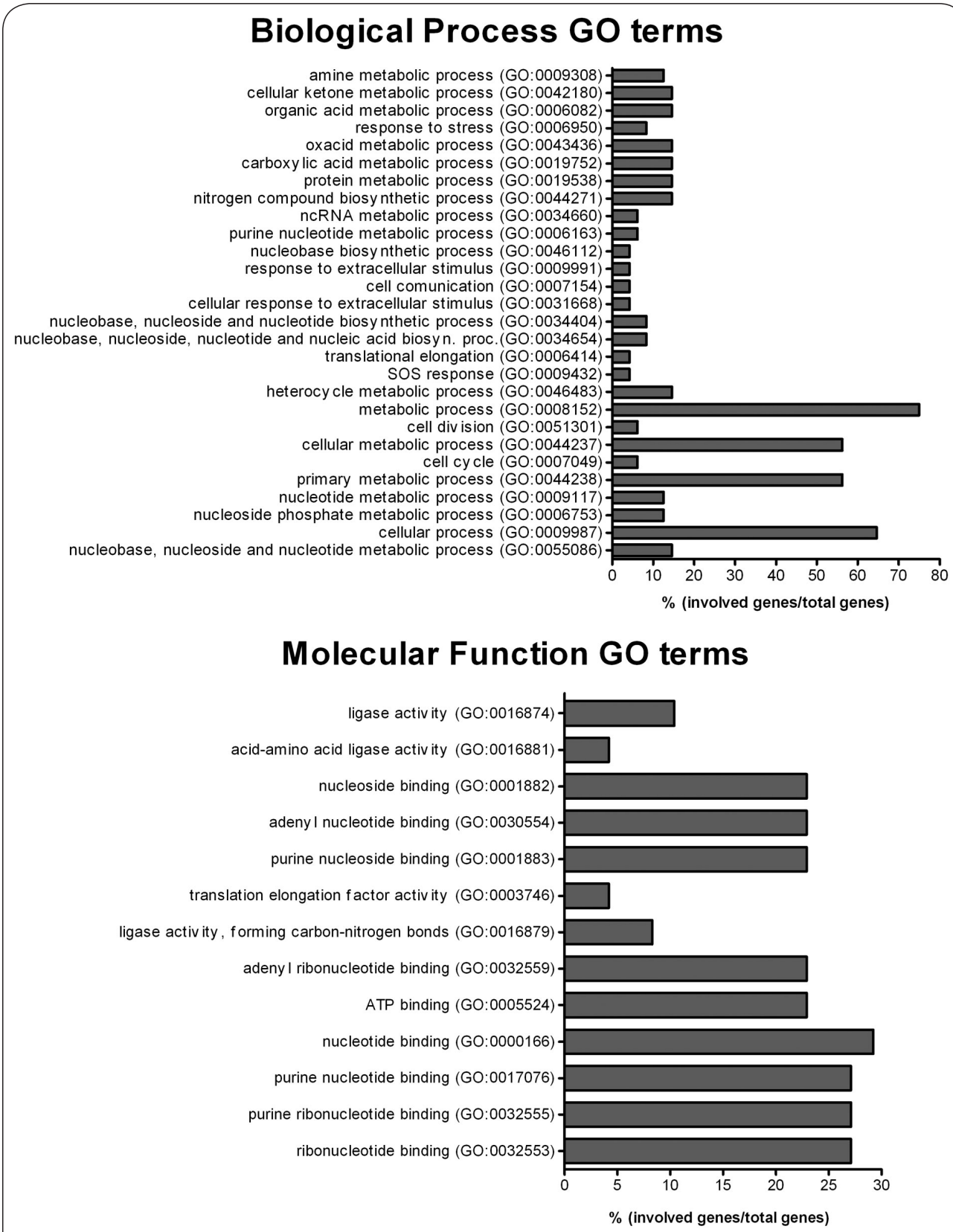

Figure 4. Enriched GO terms for Biological Process (BP) and Molecular Function (MF) through David software. Fisher's exact test was used to check for significant over-representation ($p<0.05$) of GO terms in the differentially expressed protein list against the *Burkholderia cenocepacia* genome.

ribonucleotide binding (GO:0032553), purine nucleotide binding (GO:0017076), nucleotide binding (GO:0000166) and purine nucleoside binding (GO:0001883). The statistical significant of these data strengthens the belief that the RND-4 gene deletion could affect, directly or indirectly, purine metabolic process. Overall, these findings suggest that in *B. cenocepacia* the RND-4 efflux pump biological role is not limited to drug resistance, but it seems to influence also additional cellular process.

Discussion

To our knowledge, this report constitutes the first comparative proteomic analysis of the bacterium *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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