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N-terminal-oriented proteogenomics of the marine bacterium *Roseobacter denitrificans* OCh114 using TMPP labeling and diagonal chromatography

Céline Bland[1], Erica M. Hartmann[1], Joseph A. Christie-Oleza[1,2], Bernard Fernandez[1], Jean Armengaud[1]

[1]*CEA, DSV, IBEB, Lab Biochim System Perturb, Bagnols-sur-Cèze, F-30207, France.*
[2]*University of Warwick, UK*

Corresponding author: Jean Armengaud, Laboratoire de Biochimie des Systèmes Perturbés, CEA Marcoule, DSV, iBEB, SBTN, LBSP, F-30207 BAGNOLS-SUR-CEZE, France.
Email : jean.armengaud@cea.fr**;** Tel: +33 (0)4 66 79 68 02; Fax: +33 (0)4 66 79 19 05.

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ABSTRACT

Given the ease of whole genome sequencing with next-generation sequencers, structural and functional gene annotation is now purely based on automated prediction. However, errors in gene structure are frequent, the correct determination of start codons being one of the main concerns. Here, we combine protein N-termini derivatization using (N-Succinimidyloxycarbonylmethyl)tris(2,4,6-trimethoxyphenyl)phosphonium bromide (TMPP Ac-OSu) as a labeling reagent with the COmbined FRActional DIagonal Chromatography (COFRADIC) sorting method to enrich labeled N-terminal peptides for mass spectrometry detection. Protein digestion was performed in parallel with three proteases to obtain a reliable automatic validation of protein N-termini. The analysis of these N-terminal enriched fractions by high-resolution tandem mass spectrometry allowed the annotation refinement of 534 proteins of the model marine bacterium *Roseobacter denitrificans* OCh114. This study is especially efficient regarding mass spectrometry analytical time. From the 534 validated N-termini, 480 confirmed existing gene annotations, 41 highlighted erroneous start codon annotations, five revealed totally new mis-annotated genes; the mass spectrometry data also suggested the existence of multiple start sites for eight different genes, a result that challenges the current view of protein translation initiation. Finally, we identified several proteins for which classical genome homology-driven annotation was inconsistent, questioning the validity of automatic annotation pipelines and emphasizing the need for complementary proteomic data. All data have been deposited to the ProteomeXchange with identifier PXD000337.

INTRODUCTION

Recent developments in mass spectrometry and bioinformatics have established proteomics as a common and powerful technique for identifying and quantifying proteins at a very broad scale, but also for characterizing their post-translational modifications and interaction networks (1, 2). In addition to the avalanche of proteomic data currently being reported, many genome sequences are established using next-generation sequencing, fostering proteomic investigations of new cellular models. Proteogenomics is a relatively recent field in which high-throughput proteomic data is used to verify coding regions within model genomes to refine the annotation of their sequences (2-8). Because genome annotation is now fully automated, the need for accurate annotation for model organisms with experimental data is crucial. Many projects related to genome re-annotation of microorganisms with the help of proteomics have been recently reported, such as for *Mycoplasma pneumoniae* (9), *Rhodopseudomonas palustris* (10), *Shewanella oneidensis* (11), *Thermococcus gammatolerans* (12), *Deinococcus deserti* (13), *Salmonella thyphimurium* (14), *Mycobacterium tuberculosis* (15, 16), *Shigella flexneri* (17), *Ruegeria pomeroyi* (18), and *Candida glabrata* (19), as well as for higher organisms such as *Anopheles gambiae* (20) and *Arabidopsis thaliana* (4, 5).

The most frequently reported problem in automatic annotation systems is the correct identification of the translational start codon (21-23). The error rate depends on the primary annotation system, but also on the organism, as reported for *Halobacterium salinarum* and *Natromonas pharaonis* (24), *Deinococcus deserti* (21), and *Ruegeria pomeroyi* (18) where the error rate is estimated above 10%. Identification of a correct translational start site is essential for the genetic and biochemical analysis of a protein because errors can seriously impact subsequent biological studies. If the N-terminus is not correctly identified, the protein will be considered in either a truncated or extended form, leading to errors in bioinformatic analyses (e.g., during the prediction of its molecular weight, isoelectric point, cellular localization) and major difficulties during its experimental characterization. For example, a truncated protein may be heterologously produced as an unfolded polypeptide recalcitrant to structure determination (25). Moreover, N-terminal modifications, which are poorly documented in annotation databases, may occur (26, 27).

Unfortunately, the poor polypeptide sequence coverage obtained for the numerous low abundance proteins in current shotgun MS/MS proteomic studies implies that the overall detection of N-terminal peptides obtained in proteogenomic studies is relatively low. Different methods for establishing the most extensive list of protein N-termini, grouped under the so-called "N-terminomics" theme, have been proposed to selectively enrich or improve the detection of these peptides (2, 28, 29). Large N-terminome studies have recently been reported based on resin-assisted enrichment of N-terminal peptides (30) or terminal amine isotopic labeling of substrates (TAILS) coupled to depletion of internal peptides with a water-soluble aldehyde-functionalized polymer (31-35). Among the numerous N-terminal-oriented methods (2), specific labeling of the N-terminus of intact proteins with N-tris(2,4,6-trimethoxyphenyl)phosphonium acetyl succinamide (TMPP-Ac-OSu) has proven reliable (21, 36-39). TMPP-derivatized N-terminal peptides have interesting properties for further LC-MS/MS mass spectrometry: i) an increase in hydrophobicity due to the trimethoxyphenyl moiety added to the peptides, increasing their retention times in reverse phase chromatography, ii) improvement of their ionization due to the introduction of a positively charged group, and iii) a much simpler fragmentation pattern in tandem mass spectrometry. Other reported approaches rely on acetylation, followed by trypsin digestion, and then biotinylation of free amino groups (40); guanidination of lysine lateral chains followed by N-biotinylation of the N-termini and trypsin digestion (41); or reductive amination of all free amino groups with formaldehyde preceeding trypsin digestion (42). Recently, we applied the TMPP method to the proteome of the *Deinococcus deserti* bacterium isolated from upper sand layers of the Sahara desert (13). This method enabled the detection of N-terminal peptides allowing the confirmation of 278 translation initiation codons, the correction of 73 translation starts, and the identification of non-canonical translation initiation codons (21). However, most TMPP-labeled N-terminal peptides are hidden among the more abundant internal peptides generated after proteolysis of a complex proteome, precluding their detection. This results in disproportionately fewer N-terminal validations, *i.e.*, 5% and 8% of total polypeptides coded in the theoretical proteomes of *Mycobacterium smegmatis* (37) and *Deinococcus deserti* (21) with a total of 342 and 278 validations, respectively.

An interesting chromatographic method to fractionate peptide mixtures for gel-free high-throughput proteome analysis has been developed over the last years and applied to various topics (43, 44). This technique, known as COmbined FRActional DIagonal Chromatography (COFRADIC), uses a double chromatographic separation with a chemical reaction in between to change the physico-chemical properties of the extraneous peptides to be resolved from the peptides of interest. Its previous applications include the separation of methionine-containing peptides (43), N-terminal peptide enrichment (45, 46), sulphur amino acid-containing peptides (47), and phosphorylated peptides (48). COFRADIC was identified as the best method for identification of N-terminal peptides of two archaea, resulting in the identification of 240 polypeptides (9% of the theoretical proteome) for *Halobacterium salinarum* and 220 (8%) for *Natronomonas pharaonis* (24).

Taking advantage of both the specificity of TMPP labeling, the resolving power of COFRADIC for enrichment, and the increase in information through the use of multiple proteases, we performed the proteogenomic analysis of a marine bacterium from the *Roseobacter* clade, namely *Roseobacter denitrificans* OCh114. This novel approach allowed us to validate and correct 534 unique proteins (13% of the theoretical proteome) with TMPP-labeled N-terminal signatures obtained using high-resolution tandem mass spectrometry. We corrected 41 annotations and detected five new open reading frames in the *R. denitrificans* genome. We further identified eight distinct proteins showing direct evidence for multiple start sites.

EXPERIMENTAL PROCEDURES

Preparation of protein extracts. *R. denitrificans* cells were grown in Erlenmeyer flasks containing 400 ml of Marine Broth (MB, Difco), incubated at 30 °C and agitated at 180 rpm. Cells were harvested by centrifugation at 6,000 *g* and 4 °C. Cell pellets were washed twice with ice cold 20 mM Tris/HCl buffered at pH 8.0. A quantity of 400 mg of cells (wet weight) was resuspended in 2 ml of cold buffer P consisting of 100 mM NaH_2PO_4/Na_2HPO_4 buffered at pH 8.2 (at 20 °C) and containing a protease inhibitor mixture (Complete, Roche Applied Science, six tablets in 250 ml). Cells were disrupted using a sonicator Bioblock 75042 with a total applied energy of 60 J. The sample was centrifuged for 30 min at 16,000 *g* and 4 °C to remove cellular debris. The resulting supernatant was further desalted by chromatography on two 5 ml HiTrap Desalting columns mounted in series (GE Healthcare) and operated with an Äkta Purifier 100 FPLC system (GE Healthcare) with buffer P. The sample was applied at a flow rate of 1 ml/min to columns previously equilibrated with buffer P.

N-terminal chemical labeling of *R. denitrificans* protein extracts. TMPP reagent (TMPP-AcOSu) from Fluka was dissolved at 200 mM in 20% acetonitrile (ACN)/80% H_2O (v/v) immediately before use. To each protein extract (500 µg), 15.6 µl of 200 mM TMPP-AcOSu was added (ratio of 250 TMPP molecules per protein). The mixture was incubated at room temperature for 60 min under gentle agitation. To stop the reaction, 417 µl of 1.5 M Tris/HCl buffered at pH 8.5 (ratio of 200 Tris molecules per TMPP) were added. The mixture was incubated at room temperature for 30 min and then desalted on two 5 mL HiTrap Desalting columns in series in 100 mM sodium phosphate buffered at pH 7.2 and containing 150 mM NaCl at a flow rate of 1 ml/min. After desalting, proteins were reduced with 0.05% tributylphosphine (Aldrich) at room temperature for 90 min and then alkylated with 5 mM iodoacetamide (Sigma) in the dark for 30 min at 37 °C. The mixture was again desalted as above.

Acetylation of intact proteins. Sulfo-NHS acetate (Thermo) was hydrated at a concentration of 1 M in 100 mM sodium phosphate buffered at pH 7.2 and containing 150 mM NaCl immediately before use, as it readily hydrolyses and becomes unreactive. Sulfo-NHS acetate solution was added to the TMPP-labeled protein extract to a final concentration of 10 mM and incubated 90 min at 30 °C. The mixture was desalted on two 5 mL HiTrap Desalting columns operated in series at 1 ml/min in 50 mM NH_4HCO_3 buffered at pH 7.8.

Proteolysis. The proteins were digested either with sequencing grade trypsin, chymotrypsin, or endoproteinase Glu-C (Roche Applied Science) reconstituted in 0.01% TFA, 1 mM HCl, or milliQ water, respectively, at a final concentration of 0.1 µg/ml. The enzyme solution (10 µg) was added to the proteins (2% w/w) for overnight proteolysis at 37 °C for trypsin and 25 °C for both chymotrypsin and endoproteinase Glu-C.

COFRADIC. Samples from each enzyme were dried separately using a SPD121 SpeedVac (Savant) and redissolved in 110 µl of solvent A consisting in 0.1% trifluoroacetic acid (TFA) in water/acetonitrile (98:2 vol/vol). The peptide mix (100 µl) was resolved on a reverse phase 300SB-C18 Zorbax HPLC column (4.6 mm x 150 mm, 5 µm) from Agilent Technologies operated at room temperature with an Agilent 1100 Series HPLC system equipped with G1315B diode array detector, G1322A degasser, G1311A quaternary pump, G1329A autosampler, and G1364C Analyst FC fraction collector. A linear gradient from 0 to 100% solvent B (30:70 vol/vol 0.1% TFA in water/acetonitrile) was applied over 100 min with a flow rate of 0.4 ml/min. Peptides eluting between 40 and 76 min were collected in 9 fractions of 1.6 ml each. These fractions were dried in a vacuum centrifuge. Each fraction was then redissolved in 70 µl of 50 mM sodium borate ($Na_2B_4O_7/H_3BO_3$), pH 9.2. The 2,4,6-trinitrobenzensulfonic acid (TNBS) reagent (Fluka) was diluted to 1 mM in a solution of 50 mM sodium borate ($Na_2B_4O_7/H_3BO_3$), pH 9.2. TNBS (4 nmol) was added to each fraction and incubated for 90 min at 37 °C under gentle agitation. The samples were dried before the second HPLC run. Each fraction was redissolved in 75 µl of 0.5% TFA from which 70 µl were loaded onto the reverse phase column. The solvent gradient was performed as in the primary run, and N-terminal peptides were collected in the same 4 min intervals. The 9 resulting fractions were dried, redissolved in 11 µl of 0.1% TFA, and 10 µl were analyzed using nanoLC-MS/MS.

NanoLC-MS/MS analysis. NanoLC-MS/MS experiments were performed on an LTQ–Orbitrap XL hybrid mass spectrometer (ThermoFisher) coupled to an UltiMate 3000 LC system (Dionex-LC Packings). The parameters for recording MS/MS spectra were as described previously (21). Peptide mixtures were loaded and desalted on line in a reverse phase precolumn (Acclaim PepMap 100 C18, 5 µm bead size, 100-Å pore size, 5 mm x 300 µm) from LC Packings at a flow rate of 0.3 µl/min. The previously optimized gradient (21) was applied.

Whole genome database mining. Peak lists from the LC-MS/MS raw data were generated with the MASCOT DAEMON version 2.3.2 software (Matrix Science) using the extract_msn.exe data import filter Xcalibut FT package (version 2.0.7) proposed by ThermoFisher.generated as previously described (49). To compare and optimize the different steps of the protocol, MS/MS spectra were searched against a coding domain sequence (CDS) database comprising the 4,129 polypeptides sequences annotated in the genome of *R. denitrificans* (50), totaling 1,288,451 amino acids with an average of 312 amino acids per polypeptide (NCBI download, 2010/02/17). The 22 most common keratin contaminants and the proteases used in this study were also included in the database. Searches for peptides were performed with Mascot using the following parameters: a mass tolerance of 5 ppm on the precursor ion and 0.5 Da on the MS/MS, static modification of carboxamidomethylated Cys (+57.0215), and variable modifications of oxidized Met (+15.9949), N-terminal acetylation (+42.0106), and TMPP$^+$-Ac ($C_{29}H_{35}O_{10}P$, +572.1811) or TNBS ($C_6H_2O_6N_3$, +210.9865) labels on the N-termini or Lys lateral chains. The variable modification of TMPP$^+$-Ac-Met ($C_{34}H_{44}N_1O_{11}P_1S_1$, +703.2216 amu as calculated after taking into account the additional positive charge introduced by TMPP) on the N-termini was also included; this modification, in combination with a semi-specific digestion requirement, allows the identification of translation initiation at non-AUG codons, such as when GTG codons, which normally code for valine residues, actually encode the initial methionine. Mascot will treat the resulting peptide as if the C-terminus is specifically cleaved, but not the N-terminus, which begins with a methione, contrary to the theoretically encoded valine. Mis-cleavages were set at 3 for trypsin or chymotrypsin, and 2 for endoproteinase Glu-C. All peptides with a score above its peptide identity threshold set at $p < 0.01$ with the CDS database and rank 1 were filtered by the IRMa 1.28.0 software (51). At the proteogenomic stage of genome refinement of *R. denitrificans* OCh114, the MS/MS datasets were searched against an amino acid sequence database corresponding to a six-frame stop-to-stop translation of the genome sequence and that was restricted to open reading frames (ORFs) with at least 40 amino acids. This ORF database comprised 61,652 polypeptide sequences, totaling 6,201,552 amino acids with an average of 101 amino acids per polypeptide. The sequence of the proteases and the 22 most common keratin contaminants were also included in this database. The maximum numbers of mis-cleavages were set as above. Fixed and variable modification searches were performed with the same parameters as described for the CDS database searches.

Criteria for extracting N-terminal peptides. Each nanoLC-MS/MS experiment was analyzed first with specific protease cleavages and then in semi-specific mode to identify TMPP-derivatized peptides. All peptide matches with the ORF database were filtered with the IRMA 1.28.0 software with cutoff values at $p < 0.01$. Redundant assignments to the same from the dual assignment strategy or multiple records by the mass spectrometer were reduced to a single event. Peptides having an acetylation or TMPP N-terminal label were extracted, and only non-redundant N-terminal most peptides corresponding to a possible start (ATG, GTG, and TTG codons) were conserved. Further validation was applied as follows: peptide score, coding length to total ORF length ratio greater than 0.45, similarities detected with other known proteins, and manual annotation of MS/MS spectra.

***In silico* analysis of protein sequences.** Prediction of secreted proteins was performed by analyzing proteins with evidence of multiple translational starts or incorrect annotations using the prediction tools SignalP 4.0, SecretomeP 2.0, and LipoP 1.0 as described previously (49). PSI-BLAST (http://blast.ncbi.nlm.nih.gov/blast) searches were performed using the NCBI non-redundant protein sequence database and default parameters.

Proteomic data repository. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (http://www.ebi.ac.uk/pride/) with the dataset identifier PXD000337.

RESULTS AND DISCUSSION

Enrichment of TMPP-labeled N-terminal peptides. To selectively purify N-terminal peptides, we labeled the whole proteome of *R. denitrificans* OCh114 with TMPP-Ac-OSu (**Figure 1**). After labeling with TMPP, the proteins were proteolyzed (e.g., with trypsin) generating TMPP-labeled N-terminal peptides and unlabeled internal tryptic peptides. The TMPP-labeled peptides, along with co-eluting internal peptides, were collected in an initial chromatographic separation. The co-eluting internal peptides were labeled with a second reagent, which reacts with free N-terminal amines. The newly labeled internal peptides are more hydrophobic and were thus removed in the second chromatographic step. We chose TNBS as the second labeling reagent, and to avoid any labeling of free amino groups from lateral chains with this second reagent that would inhibit enrichment, we introduced a preliminary protection step by acetylating lysine lateral chains with sulfo-NHS-acetate.

To test the efficiency of this acetylation step, we analyzed an acetylated tryptic digest ("Ta" in **Table 1**) and compared the results with those obtained with an equivalent non-acetylated sample ("Tb" in **Table 1**). Both fractions were analyzed in a single nanoLC-MS/MS run, and MS/MS spectra were searched against the CDS database. A complete list of assigned peptides and proteins is given in **Supplementary Table S1**. No acetylated peptides were detected when the sample was not treated with sulfo-NHS-acetate ("Tb" in **Table 1**), whereas the acetylated sample showed 73% protection of lysine residues ("Ta" in **Table 1**). In the acetylated sample, 113 distinct N-terminal peptides matching annotated proteins were found, corresponding to 89 non-redundant polypeptides. Of these, 65 were detected with their N-terminus labeled with TMPP; 23 were acetylated at the N-terminus, and one was unmodified. Although useful for protecting lysine residues before TNBS labeling, acetylation introduces steric hindrance (42, 52), which inhibits trypsin recognition of the lysine residue as a cleavage site and thus inhibits digestion. Without acetylation, 18% of identified peptides had one mis-cleavage; with acetylation, the percentage of peptides with one mis-cleavage increases to 31. Furthermore, 4% of peptides were identified with three mis-cleavages. In addition to having more mis-cleavages, fewer peptides ending with a lysine residue were found in the acetylated sample. Only 18% of peptides ended with a lysine, compared to 42% in the non-acetylated sample (**Supplementary Table S1**).

We resolved the TMPP-labeled and acetylated peptides on a reverse-phase column and collected peptides eluting between 44 and 60 min in four fractions of 1.6 ml each. These fractions were then analyzed using nanoLC-MS/MS. Data from the four runs ("T1" in **Table 1**) resulted in the identification of 3,449 different peptides (**Table 1** and **Supplementary Table S1**). Of these, 325 were N-terminal peptides. As expected, increasing the number of spectra acquired in this experiment compared to our previous analysis ("Ta" in **Table 1**) led to a higher number of peptides identified. We then modified the same four fractions with TNBS and subjected them to diagonal reverse-phase chromatography. The four resulting fractions were then analyzed by nanoLC-MS/MS. This dataset ("T2" in **Table 1**) yielded less than half of the total identifications from Data "T1" (1,559) but a higher number of N-terminal peptides (386). After the first chromatography step, 9% of identified peptides corresponded to N-termini, whereas after the second chromatography step, they increased to 25%, showing a clear enrichment of these specific peptides. Only 25 TNBS-labeled peptides were found in these four fractions ("T2" in **Table 1**) and none corresponded to the N-terminus of a protein. As expected, fractions eluting with a delay contained a higher ratio of TNBS-labeled internal peptides (data not shown). A total of 291 unique proteins were detected through their N-terminal peptides, compared to 230 found without the intermediate COFRADIC step. Therefore, this method is efficient at enriching N-terminal peptides.

Spectral assignment using the CDS and six-frame ORF databases. Using nanoLC-MS/MS, we analyzed all the COFRADIC fractions from the tryptic digest, generating 93,545 MS/MS spectra (**Supplementary Table S2**). After merging the search results and removing the peptides assigned to ambiguous loci, contaminants, and protease autolysis, 61,849 MS/MS spectra assigned to tryptic or semi-tryptic peptides remained. These peptides correspond to 1,556 potential N-terminal sequences with the conventional translational start codons ATG, GTG, and TTG (**Supplementary Table S3**). MS/MS data acquired from chymotrypsin and endoproteinase Glu-C digests were processed as for the tryptic digest, allowing 3 and 2 mis-cleavages, respectively. After processing, samples from these two enzymes yielded an additional 24,052 unique MS/MS spectra. In total, 2,603 peptides beginning with the possible start codons ATG, GTG, or TTG (**Supplementary Table S3**) were found, corresponding to 840 non-redundant proteins. From the whole peptide pool, the vast majority (1,556) arose from trypsin, with 473 and 574 generated by chymotrypsin and endoproteinase Glu-C, respectively. The false discovery rate for peptide assignment, estimated using the same search strategy with a decoy database, was less than 1% with both CDS and six-frame ORF databases. Decoy databases were constructed using the “decoy” function in the Mascot Daemon and consisted of reversed amino acid sequences from the target databases.

Validation of TMPP-labeled N-terminal peptides. For automatic validation of N-terminal start sites we took advantage of data redundancy arising from differences in terms of mis-cleavages, methionine oxidation, different proteolytic enzymes (*i.e.*, trypsin, chymotrypsin, and endoproteinase Glu-C), and partial removal of the initial methionine. Chymotrypsin specifically hydrolyzes peptide bonds at the C-termini of tyrosine, phenylalanine, and tryptophan; leucine, methionine, alanine, aspartic acid, and glutamic acid are cleaved at a lower rate (53). Endoproteinase Glu-C cleaves peptide bonds at the C-terminal of glutamic acid and, at a much lower rate (3,000 fold), aspartic acid (54). When the same N-terminal sequence is observed following digestion with two or more enzymes, these multiple pieces of evidence corroborate valid protein N-termini. Of the total 2,603 N-terminal peptide identifications matching previously annotated proteins, 349 were automatically validated based on redundant identifications. The false discovery rate for N-terminal peptide assignment was estimated to be less than 1% using the same approach and the ORF target-decoy database. Most of the detected proteins (66%) were identified from their N-terminal initiation peptides recorded with only one protease, while 25% and 9% of the proteins were detected with a combination of two or all three proteases, respectively. Of those detected with at least two proteases, more proteins are identified with the combination of trypsin and endoproteinase Glu-C (70 proteins), than with trypsin and chymotrypsin (45 proteins) or endoproteinase Glu-C and chymotrypsin (8 proteins). Protease mis-cleavages allowed the corroboration of sequences pointing at the same N-terminal initiation, more so even than parallel proteolysis with different enzymes. Here, trypsin was responsible for most of the mis-cleavages, mainly due to proteolysis inhibition by lysine acetylation. The 491 remainder proteins seen with only one peptide could be further evaluated. We validated 131 protein identifications produced from unique peptides after manual inspection of the corresponding MS/MS spectra. Criteria for inspection included the detection of at least one of the three described TMPP reporter ions, the *a1* and *b1* ions, and the presence of *a* ions; only peptides in the top third of the peptide list ranked by ion score, *i.e.*, above 23, were considered (**Supplementary Table S4**). As an example, **Figure 2** shows the MS/MS spectrum attributed to the N-terminus of YP_684061.1, a hemolysin-like protein. The presence of the TMPP-CHCO$^+$ reporter ion, the *b1* ion, the presence of 3 *a* ions, and the almost complete *b* ion series

(*b_1*-*b_{11}*) justifies the assignment of this MS/MS spectrum to the GDNDGSSDAAQSAR peptide.

Of the 480 resulting N-termini that matched previous annotations, 63% were exclusively detected with a cleaved initial methionine, while 9% showed partial removal of this N-terminal amino acid. Methionine removal occured when alanine, serine, and threonine, and to a lesser extent proline, glycine, valine, and asparagine, are in the second position of the N-terminal peptide sequence, confirming what has been reported previously (55, 56). Incomplete methionine excision was mainly observed with threonine or asparagine in second position of the polypeptide. Their longer lateral chain compared to the other cleavable residues may explain this pattern.

Re-annotation of the *R. denitrificans* OCh114 genome. A total of 41 proteins were identified with N-termini different from their initial annotation (**Supplementary Table S4**). Of these 41 proteogenomic re-annotations, 18 are longer than previously annotated and 23 are shorter. **Figure 3** shows eight different peptides corresponding to the correct N-terminus of YP_680835.1, a Rho transcription termination factor, for which 10 residues were missing in the primary annotation. The true start codon (ATG) is only conserved in the closely related strain *R. litoralis* OCh149 and not in other *Roseobacter* strains (**Figure 3)**. The previously annotated start site (GTG) was likely erroneously determined based on similarity with the ATG start codon of other homologs.

The gene coding for the fructose-1,6-bisphosphate aldolase (YP_682542.1) was also mis-annotated, as evidenced by four TMPP-labeled peptides originating six amino acids upstream of the previous annotation. Unlike the previous example, the correct start site of this protein is more conserved in closely related homologs than the initial annotation. Ten different peptides indicated a correction of the initiation codon for the 50S ribosomal protein L21 (YP_682897.1) 174 nucleotides downstream from the previously annotated start site, which produces a 58 amino acid truncation. For the transcription elongation factor GreA (YP_683583.1), nine peptides indicated a well-defined start site 468 nucleotides downstream of the current CDS start site (half of the previously annotated sequence). Similarly, TMPP-labeled peptides also indicated a mis-annotation for the DNA-directed RNA polymerase subunit alpha (YP_681761.1) and the polyamine ABC transporter, ATP-binding protein (YP_680591.1). In this later case, the mass spectrometry-certified translation start site is a TTG codon, which is strictly conserved in closely related homologs.

Evidence of multiple translational starts in eight proteins. Multiple translational start sites were found for eight proteins, as evidenced by several TMPP-labeled peptides (**Supplementary Table S4**). Alternative start sites both up- and downstream of the original annotated starts were observed. For the ATP-dependent Clp protease proteolytic subunit (YP_683070.1), 16 different TMPP-labeled peptides indicated three start sites: the current CDS annotation starting at position 2,727,465 of the chromosome (confirmed with seven peptides), and two other translational start sites located at the positions 2,727,441 and 2,727,426 confirmed with eight and one peptides, respectively (**Figure 4**). These start sites correspond to gene products of 208, 200, and 195 amino acids in length, respectively. PSI-BLAST indicated that all three start codons are strictly conserved in closely related homologs. The N-terminus of Clp proteolytic subunit is highly conserved in general, as the 20 most N-terminal amino acids coordinate binding with the ATPase subunit of this multimeric protease (57). The N-terminus adopts different conformations to control this coordination and to gate the proteolytic chamber (58), and truncations of the N-terminus allow degradation of large substrates in the absense of the ATPase subunit (59) and increase the initial degradation rate while stabilizing the acyl-enzyme intermediate (60). In *E. coli*, ClpP is first synthesized with a 14-amino acid signal peptide; however, the immature polypeptide has a half-life of approximately 2 min, and the mature product is then extremely stable (61). The N-termini of ClpP identified here in *R. denitrificans* Och114 correspond to the mature N-terminus of the *E. coli* homolog, 8 amino acids upstream, and 5 amino acids downstream. The existence of multiple isoforms of this N-terminus could be indicative of a prolonged half-life of the immature polypeptide, or it could play a role in controlling the rate of degradation and/or the selectivity of ClpP in this strain.

An alternative translational start was also detected for the ferredoxin-NADP reductase YP_683175.1 (**Supplementary Figure S1**), corresponding to the original annotation and a 6-amino acid truncation. The ferredoxin-NADP oxidoreductase gene *petH* from the cyanobacterium *Synechocystis* sp. Strain PCC6803 has three transcriptional start sites, producing two translation products corresponding to a 413- and a 300-amino acid isoforms of this protein, which are differentially expressed in response to nitrogen availability (62). In both *R. denitrificans* Och114 and *Synechocystis*, the truncated N-terminal region is outside of the conserved domains. In *Synechocystis*, this region is responsible for interaction with phycobilisome, and thus binding is inhibited in its absence. Similarly, the 6-amino acid N-terminal region of this ferredoxin-NADP reductase in *R. denitrificans* Och114 may be used to influence its interaction with certain partners.

Similarly, two translational starts were detected for the recombinase A (YP_682935.1), one of which corresponds to the original annotation and the other 7 amino acids downstream. The agmatinase (YP_682939.1) was observed with one start site corresponding to the original annotation and another 7 amino acids upstream. The dnaK suppressor protein YP_683008.1 similarly had a start site 11 amino acids upstream, in addition to the original annotation. All of the alternative start sites leave the conserved functional domain intact. The hypothetical protein RD1_1231 (YP_681565.1), for which 17 TMPP-labeled peptides were recorded (**Supplementary Figure S2** and **Table S4**), was also observed with two start sites: 12 peptides corresponded to the CDS annotation and 5 corresponded to a start site 9 nucleotides downstream. Alternative in-frame translation starts have been described for several bacterial proteins, such as the outer membrane protease IcsP from *Shigella flexneri* (63), and the type IV pili component PilA from *Geobacter sulfurreducens* (64).

Evidence for a protein with a non-canonical translational start codon. Two starts sites were found for the iojap-like protein (YP_681006.1). Two TMPP-labeled peptides corresponding to the N-terminus of the existing annotation were observed; another five peptides corresponded to the unconventional start codon CTG at position 602,762 of the chromosome, implying a 16-amino acid prolongation. The annotated N-termini of homologs of this protein from closely-related bacteria are heterogeneous, but the CTG codon is strictly conserved among these genomes (**Figure 5**). This non-canonical alternative start codon may therefore be common to all of these organisms. Because a serine residue is found in second position, the initial methionine should be systematically removed, which is in agreement with the observed TMPP-labeled peptides. The human iojap ortholog has been found associated with the large subunit of the mitochondrial ribosome in humans (65), while the plant ortholog has been shown to be vital for chloroplast ribosome biogenesis (66), and several bacterial orthologs (RsfA/YbeB) are conserved ribosomal silencing factors (67).

Discovery of five un-annotated CDS. Five previously un-annotated genes in the genome of *R. denitrificans* OCh114 were detected during this study at loci 331,094-330,567 (frame -1), 338,913-339,212 (frame 3), and 1,376,295-1,377,065 (frame 3) on the chromosome and loci 4,139-4,615 (frame 2) and 4,012-3,548 (frame -3) on the pTB4 plasmid (**Supplementary**

Table S5). The coordinates of these genes do not overlap with those of previously annotated genes, potential promoters and Shine-Dalgarno sequences exist upstream of each coding sequence, and the corresponding peptides could not be assigned to any other gene. The ORF located at the position 331,094-330,567 on the chromosome was validated with two different peptides obtained during the semi-trypsin and semi-endoproteinase Glu-C analysis; an annotated spectrum from the semi-triptic peptide, as well as the predicted sequence of the entire gene and corresponding product, is shown in **Figure 6**. This orphan CDS has no homologs in the NCBI database and thus no function could be assigned. The novel gene at locus 338,913-339,212 on the chromosome was observed with two translation start sites, both of which were detected by two different TMPP-labeled peptides (**Supplementary Table S5**). The closest homologue to this protein was a "hypothetical protein" found in *Roseobacter sp.* AzwK-3b, and in this case only the second translation initiation site is given. The novel gene at locus 1,376,295-1,377,065 on the main chromosome was identified by two peptides. Although not previously annotated in the *R. denitrificans* OCh114 genome, a homolog of this protein is present in the closely related strain *R. litoralis* OCh149, where it is defined as a glutamate/glutamine/aspartate/asparagine transport ATP-binding protein BztD. Two additional ORFs at positions 4,139-4,615 and 4,012-3,548 on the plasmid pTB4 were identified with one N-terminal TMPP-labeled peptide each. Again, low sequence similarities with other proteins were noted for the two polypeptides and no function could be predicted.

Concluding remarks. By combining the TMPP labeling of protein N-termini (to increase the ionizability and simplify fragmentation of peptides during mass spectrometry) and the COFRADIC enrichment of such labeled peptides (to remove internal non-informative peptides), we characterized 534 N-termini: 480 matching the existing annotation, 41 corrections to the previous annotation, 5 previously un-annotated proteins, and 8 proteins with multiple translational starts. Of the observed proteins, almost 10% were incorrectly annotated in terms of translational start codon. In some cases, classical homology-driven annotation of genomes may lead to incorrect annotation, such as observed for the transcription termination factor Rho, where the corrected start site was not conserved in different *Roseobacter* strains. This questions the use of ortho-proteogenomics, as first proposed by Gallien and coworkers (37), which uses mass spectrometry-based empirical evidence from one strain to evaluate other related organisms. Indeed, this observation emphasizes the need for empirical evaluation of every sequenced organism for maximum annotation accuracy. Furthermore, multiple translational start sites were observed for several proteins. While it is widely acknowledged for eukaryotes that a single gene may code for several protein variants (68), the same generality has yet to be demonstrated for prokaryotes. This proteome-wide proteomic analysis of *R. denitrificans* OCh114 shows that such phenomena exist on a moderate scale in bacteria, an observation that challenges the conventional idea that each gene encodes one unique protein sequence in bacteria (69).

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FIGURE LEGENDS

Figure 1. Strategy applied for N-termini enrichment. Proteins are first labeled with TMPP on their N-terminus before reduction and alkylation of cysteines. All free amino groups are further protected through an acetylation step. Protein digestion generates a mixture containing both N-terminal TMPP-labeled peptides and non-labeled internal peptides that is resolved by C18 reverse-phase liquid chromatography. Fractions with eluted peptides are isolated and treated separately with TNBS reagent, which reacts with free amino groups of internal peptides. A second chromatographic run carried out in exactly the same conditions is performed to resolve TNBS-altered internal peptides (higher hydrophobicity) from the non-modified TMPP-labeled N-terminal peptides eluted at the same retention time. The chromatogram of fraction F52 after the second chromatography is shown. The N-terminal peptides of interest are further collected and analyzed by tandem mass spectrometry.

Figure 2. Manual verification of a TMPP-modified peptide. The fragmentation spectrum of the N-terminal TMPP-modified peptide [GDNDGSSDAAQSAR] assigned to the protein YP_684061.1 is shown. The almost complete *b* ion series and the reporter ion TMPP-CHCO$^+$ give a high confidence in the assignment of this MS/MS spectrum. The *a* ions series and the reporter ion are indicated in red.

Figure 3. Re-annotation of the transcription termination factor Rho from *R. denitificans* OCh114. Synthesis of the protein YP_680835.1 was predicted to start from a GTG initiation codon based on homologous sequences found in other members of the *Roseobacter* clade. However, N-terminal peptides demonstrated that the real start site was 30 nucleotides upstream from the initial annotation (ATG codon, underlined and in bold red). This codon was only conserved in the closest-related strain *R. litoralis* Och149. Previously annotated codons are shown in red and their counterparts in other sequences are in blue. Acetylated residues are indicated by a star.

Figure 4. Multiple translation start sites of the ATP-dependent Clp protease proteolytic subunit. Three alternative start sites were recorded by multiple detected N-terminal peptides (represented as blue lines) for protein YP_683070.1. A multiple sequence alignment with closely-related homologues demonstrates the heterogeneity of initiation sites and how this protein should be re-annotated in multiple species as all three codons are strictly conserved

(ATG codons underlined and in bold red). The translated polypeptides from the previous annotation and from the new corrected annotation are labeled 1 and 2, respectively.

Figure 5. Re-annotation of the iojap-like protein initiated with a non-conventional start codon. The sequence alignment of the iojap-like protein (YP_681006.1) is shown with closely-related bacteria. The translated polypeptide from the previous annotation and from the new corrected annotation are labeled 1 and 2, respectively. Previously annotated codons are shown in red and their counterparts in other sequences are in blue. The two alternative start codons are indicated in bold red and underlined. Blue lines represent the N-terminal peptide sequences identified in *R. denitrificans* OCh114. Initiation heterogeneity is demonstrated and re-annotation of this protein in closely-related species is suggested in red.

Figure 6. Discovery of a novel gene from *Roseobacter denitrificans* OCh114. MS/MS spectrum of the TMPP-modified peptide [MENVVDETIAGLR] which indicated the N-terminus of the newly-discovered gene encoded on the chromosome in frame -1 between positions 331,094 and 330,567 (nucleotide sequence displayed). The peptide detected by tandem mass spectrometry is highlighted in bold red in the sequence. On the fragmentation spectrum, the reporter ion TMPP-CHCO$^+$ and the *a* ion series are shown in red. The *b* ions series is shown in black. The stop codon is indicated with a star symbol.

Table 1. TMPP assignment results from the CDS database searches[1].

	Number of assigned MS/MS spectra	Number of non-redundant peptides (unique peptide sequences and modifications)	Number of N-terminus peptide signatures	Number of unique proteins with an N-terminus signature	Number of proteins with labeled TMPP peptides	% of acetylated lysines on non-redundant peptides
Tb	4,183	2,049	128	102	98	0%
Ta	3,461	1,731	113	89	65	73%
T1	10,296	3,449	325	230	165	70%
T2	3,529	1,559	386	291	212	83%

[1] Peptide information is indicated in **Supplementary Table S1**.

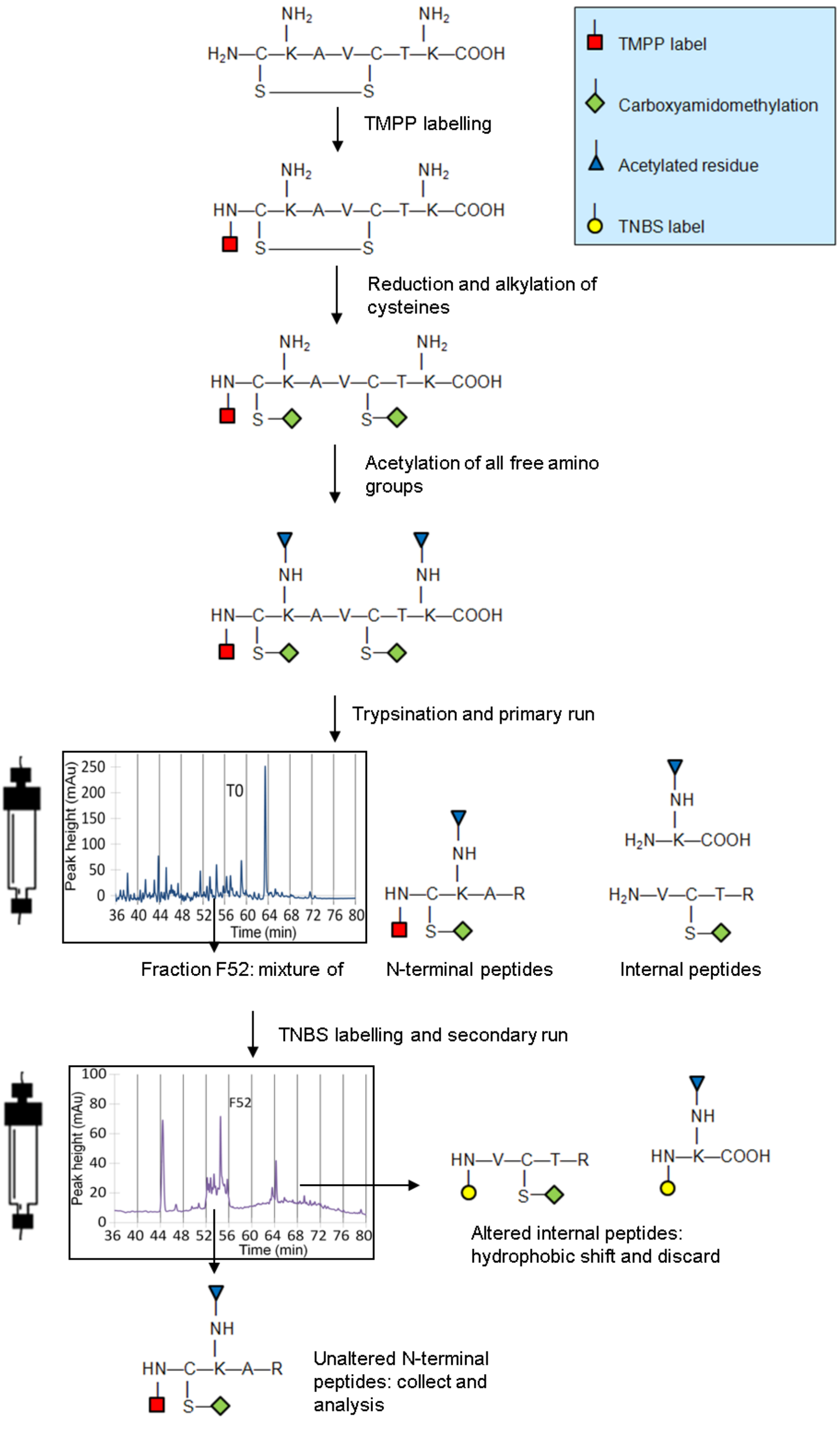
H_2N—C—K—A—V—C—T—K—COOH
NH_2
S
TMPP labelling
HN—C—K—A—V—C—T—K—COOH
Reduction and alkylation of cysteines
Acetylation of all free amino groups
NH
Trypsination and primary run
Peak height (mAu)
250
200
150
100
50
0
36 40 44 48 52 56 60 64 68 72 76 80
Time (min)
T0
Fraction F52: mixture of
N-terminal peptides
Internal peptides
HN—C—K—A—R
H_2N—K—COOH
H_2N—V—C—T—R
TNBS labelling and secondary run
100
80
60
40
20
0
F52
HN—V—C—T—R
HN—K—COOH
Altered internal peptides: hydrophobic shift and discard
Unaltered N-terminal peptides: collect and analysis
TMPP label
Carboxyamidomethylation
Acetylated residue
TNBS label

Figure 1

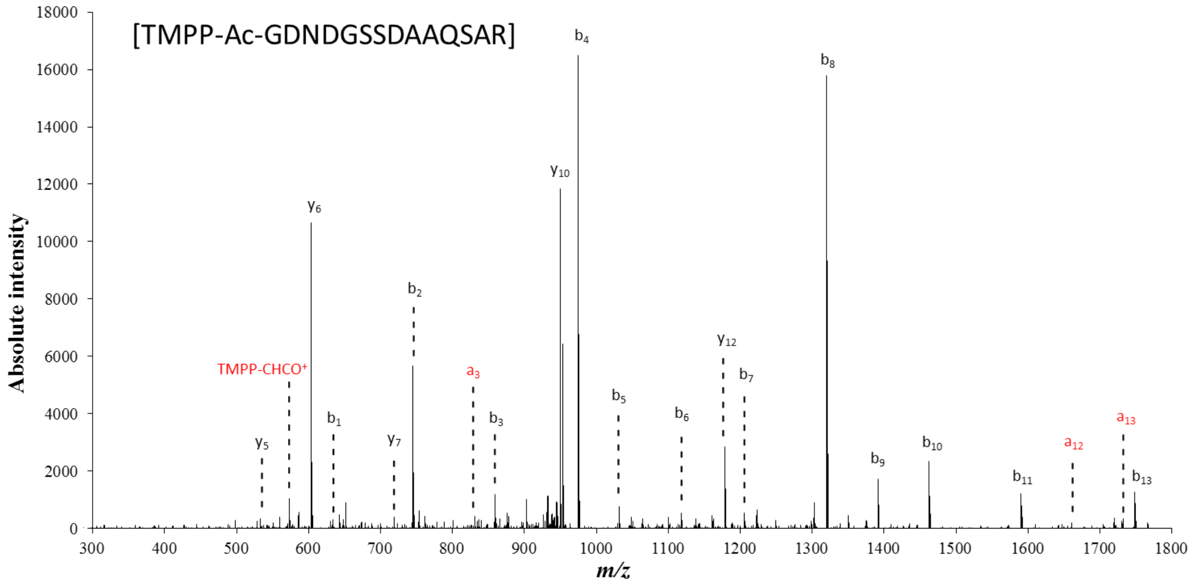
[TMPP-Ac-GDNDGSSDAAQSAR]
Absolute intensity
m/z
0
2000
4000
6000
8000
10000
12000
14000
16000
18000
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
b4
b8
y10
y6
b2
y12
b7
TMPP-CHCO+
a3
b5
b6
b1
b3
y7
y5
a13
a12
b9
b10
b11
b13

Figure 2

```
     >gi|110677828:426469-426368 Roseobacter denitrificans OCh 114, complete genome
    ATGACATCCCAAGACACTGATACCGAAACGGTG---ACGGAAACGTTGAATCTGGCAGATCTCAAGCGGAAGAGTCCCAAGGATTTGCTGTCGATGGCAGAAGAT
1                                    V  -  T  E  T  L  N  L  A  D  L  K  R  K  S  P  K  D  L  L  S  M  A  E  D
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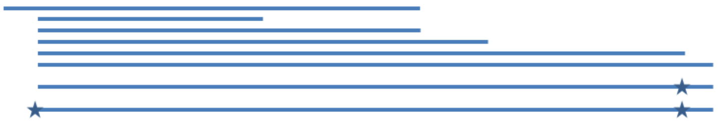

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2    M  T  S  Q  D  T  D  T  E  T  V  -  T  E  T  L  N  L  A  D  L  K  R  K  S  P  K  D  L  L  S  M  A  E  D

     >gi|339505606:4211941-4212042 Roseobacter litoralis OCh 149, complete genome
    ATGACATCTCAAGACACTGATACCGAAACGGTT---ACAGAAACGCTGAACCTGGCGGACCTTAAGCGTAAGAGTCCGAAGGATTTGTTGTCGATGGCTGAAGAT
1    M  T  S  Q  D  T  D  T  E  T  V  -  T  E  T  L  N  L  A  D  L  K  R  K  S  P  K  D  L  L  S  M  A  E  D

     >gi|163745112:974464-974378 Oceanibulbus indolifex HEL-45, complete genome
    GCATTCTGCTGCGGC * ACAGGATGCTTCATGACAACCGAAACGCTGAACCTCGCTGATCTCAAGGCGCAGACGCCGAAAAACCTGCTCGCCATGGCCGAAGAG
1                                    M  T  T  E  T  L  N  L  A  D  L  K  A  Q  T  P  K  N  L  L  A  M  A  E  E

     >gi|83942026:894536-894640 Sulfitobacter sp. EE-36, complete genome
    CGCATTCCGGGATCGGAACAGGATATTTCCATGACAACTGAAACACTTAACCTTGCCGATCTAAAGGCGCAGACGCCCAAGAATCTTCTGGCGATGGCCGAAGAG
1                                    M  T  T  E  T  L  N  L  A  D  L  K  A  Q  T  P  K  N  L  L  A  M  A  E  E

     >gi|260431963:1047597-1047701 Silicibacter lacuscaerulensis ITI-1157, complete genome
    GCCAAGGTCCGTATCATCATGACAACCGAAACTATCACTGAATCCCTGAACCTCGCGGATCTCAAAGCCAAAAGCCCCAAAGACCTTCTGGCCATGGCCGAAGAA
1                        M  T  T  E  T  I  T  E  S  L  N  L  A  D  L  K  A  K  S  P  K  D  L  L  A  M  A  E  E

     >gi|56698705:4106811-4106915 Ruegeria pomeroyi DSS-3, complete genome
    CGCCACAGGGCGGCAGAGGGTCCGTATACGATGACGATCGAATCCCTGAATCTTGCCGATCTCAAGGCAAAGAGCCCCAAGGACCTGCTGTCCATGGCCGAGGAG
1                                    M  T  I  E  S  L  N  L  A  D  L  K  A  K  S  P  K  D  L  L  S  M  A  E  E

     >gi|126738506:118695-118606 Roseobacter sp. SK09-2-6, complete genome
    AAATTGCTTGGG * AAGGGCCATATATTAATGACCGTCGATTCCCTAAATCTGTCCGATCTTAAGGCGAAAAGCCCCAAAGCACTTTTGGCTATGGCTGAAGAG
1                                    M  T  V  D  S  L  N  L  S  D  L  K  A  K  S  P  K  A  L  L  A  M  A  E  E
```
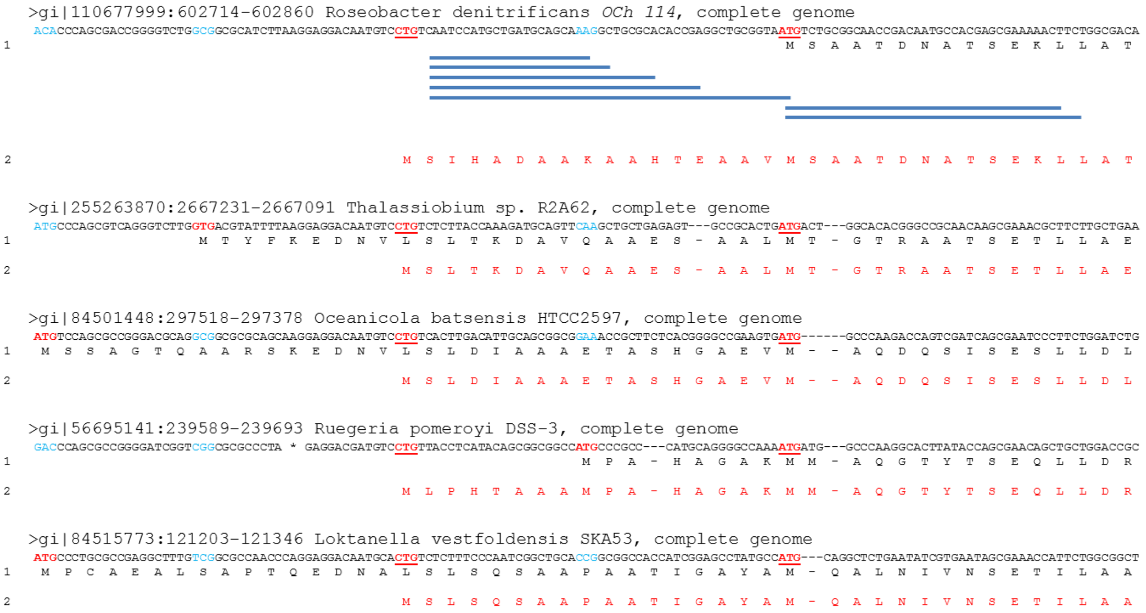

Figure 3

```
    >gi|110680063:2727489-2727385 Roseobacter denitrificans OCh 114, complete genome
    CGACGCAGCAGACAGGCATTCCACATGAAAGATCCGATTGAAACCTACATGAAC---CTTGTTCCCATGGTCGTGGAGCAAACCAGCAGGGGCGAGCGTGCATATGAC
1                            M  K  D  P  I  E  T  Y  M  N  -  L  V  P  M  V  V  E  Q  T  S  R  G  E  R  A  Y  D

    >gi|339503121:1635673-1635777 Roseobacter litoralis OCh 149, complete genome
    CGAGGCAGCAGACAGGCATTCCACATGAAAGACCCGATTGAAACATACATGAAC---CTTGTTCCCATGGTCGTGGAACAAACCAGCAGAGGCGAACGCGCCTACGAC
1                            M  K  D  P  I  E  T  Y  M  N  -  L  V  P  M  V  V  E  Q  T  S  R  G  E  R  A  Y  D

    >gi|99081368:1631417-1631310 Ruegeria sp. TM1040, complete genome
    ATGATCCCAAGACAGGCAGGTCCAATGATTGATCCAACAGAAACATACATGAACACACTTGTGCCGATGGTCGTTGAGCAGACCAGCCGGGGCGAGCGCGCATATGAC
1    M  I  P  R  Q  A  G  P  M  I  D  P  T  E  T  Y  M  N  T  L  V  P  M  V  V  E  Q  T  S  R  G  E  R  A  Y  D

2                            M  I  D  P  T  E  T  Y  M  N  T  L  V  P  M  V  V  E  Q  T  S  R  G  E  R  A  Y  D

    >gi|16374918:2020-1913 Phaeobacter gallaeciensis 2.10, complete genome
    ACAATTCAAAGCAGGCAGGTCAATATGGTCGATCCAAGAGAAACATACATGAACACGCTGGTGCCGATGGTGGTGGAACAGACCAGCCGCGGCGAACGCGCCTATGAT
1                                                    M  N  T  L  V  P  M  V  V  E  Q  T  S  R  G  E  R  A  Y  D

2                            M  V  D  P  R  E  T  Y  M  N  T  L  V  P  M  V  V  E  Q  T  S  R  G  E  R  A  Y  D

    >gi|84515416:189211-189315 Loktanella foldensis SKA53, complete genome
    TCCGTCCAGCAGAGAGGCATGAACATGCAAGACCCCGTACAAACCTATATGAAC---CTTGTTCCCATGGTCGTGGAACAGACCGCGCGTGGCGAGCGTGCCTATGAC
1                                                    M  N  -  L  V  P  M  V  V  E  Q  T  A  R  G  E  R  A  Y  D

2                            M  Q  D  P  V  Q  T  Y  M  N  -  L  V  P  M  V  V  E  Q  T  A  R  G  E  R  A  Y  D

    >gi|254467045:1977791-1977684 Rhodobacterales bacterium Y4l, complete genome
    CATAAACAACGCAGGCAGGTCGAAATGATTGATCCCAGAGAAACGTACATGAACACCCTGGTCCCGATGGTGGTTGAGCAGACCAGCCGGGGCGAGCGGGCCTATGAC
1                                                    M  N  T  L  V  P  M  V  V  E  Q  T  S  R  G  E  R  A  Y  D

2                            M  I  D  P  R  E  T  Y  M  N  T  L  V  P  M  V  V  E  Q  T  S  R  G  E  R  A  Y  D

    >gi|83949690:247792-247685 Roseovarius nubinhibens ISM, complete genome
    AGAAACTTCAGCAGGCAGGCGATTATGAAAGACCCTCTTGAGACGTATATGAACACGCTCGTGCCCATGGTGGTCGAGCAGACAAGCCGTGGCGAACGCGCCTATGAC
1                                                    M  N  T  L  V  P  M  V  V  E  Q  T  S  R  G  E  R  A  Y  D

2                            M  K  D  P  L  E  T  Y  M  N  T  L  V  P  M  V  V  E  Q  T  S  R  G  E  R  A  Y  D

    >gi|84686590:132662-132555 Maritimibacter alkaliphilus HTCC2654, complete genome
    GGGAACAGTCAGAGGCAGGCGACCATGAAAGACCCCATTGATACTTATATGAACACCCTCGTACCGATGGTCGTGGAACAGACCAGCCGGGGCGAACGGGCCTATGAC
1                                                                      M  V  V  E  Q  T  S  R  G  E  R  A  Y  D

2                            M  K  D  P  I  D  T  Y  M  N  T  L  V  P  M  V  V  E  Q  T  S  R  G  E  R  A  Y  D

    >gi|254512409:247165-247058 Rhodobacteraceae bacterium KLH11, complete genome
    CATCGCAAAGCAGGCAGGTTCCGAATGTTCGATCCAGTTGACACCTATATGAACACACTCGTCCCTATGGTCGTCGAGCAGACCAGCCGGGGCGAGCGCGCGTATGAT
1                                                                      M  V  V  E  Q  T  S  R  G  E  R  A  Y  D

2                            M  F  D  P  V  D  T  Y  M  N  T  L  V  P  M  V  V  E  Q  T  S  R  G  E  R  A  Y  D
```

Figure 4



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    >gi|110677999:602714-602860 Roseobacter denitrificans OCh 114, complete genome
     ACACCCAGCGACCGGGGTCTGGCGGCGCATCTTAAGGAGGACAATGTCCTGTCAATCCATGCTGATGCAGCAAAGGCTGCGCACACCGAGGCTGCGGTAATGTCTGCGGCAACCGACAATGCCACGAGCGAAAAACTTCTGGCGACA
1                                                                                                       M  S  A  A  T  D  N  A  T  S  E  K  L  L  A  T

2                                                    M  S  I  H  A  D  A  A  K  A  A  H  T  E  A  A  V  M  S  A  A  T  D  N  A  T  S  E  K  L  L  A  T

    >gi|255263870:2667231-2667091 Thalassiobium sp. R2A62, complete genome
     ATGCCCAGCGTCAGGGTCTTGGTGACGTATTTTAAGGAGGACAATGTCCTGTCTCTTACCAAAGATGCAGTTCAAGCTGCTGAGAGT---GCCGCACTGATGACT---GGCACACGGGCCGCAACAAGCGAAACGCTTCTTGCTGAA
1                          M  T  Y  F  K  E  D  N  V  L  S  L  T  K  D  A  V  Q  A  A  E  S  -  A  A  L  M  T  -  G  T  R  A  A  T  S  E  T  L  L  A  E

2                                                    M  S  L  T  K  D  A  V  Q  A  A  E  S  -  A  A  L  M  T  -  G  T  R  A  A  T  S  E  T  L  L  A  E

    >gi|84501448:297518-297378 Oceanicola batsensis HTCC2597, complete genome
     ATGTCCAGCGCCGGGACGCAGGCGGCGCGCAGCAAGGAGGACAATGTCCTGTCACTTGACATTGCAGCGGCGGAAACCGCTTCTCACGGGGCCGAAGTGATG------GCCCAAGACCAGTCGATCAGCGAATCCCTTCTGGATCTG
1     M  S  S  A  G  T  Q  A  A  R  S  K  E  D  N  V  L  S  L  D  I  A  A  A  E  T  A  S  H  G  A  E  V  M  -  -  A  Q  D  Q  S  I  S  E  S  L  L  D  L

2                                                    M  S  L  D  I  A  A  A  E  T  A  S  H  G  A  E  V  M  -  -  A  Q  D  Q  S  I  S  E  S  L  L  D  L

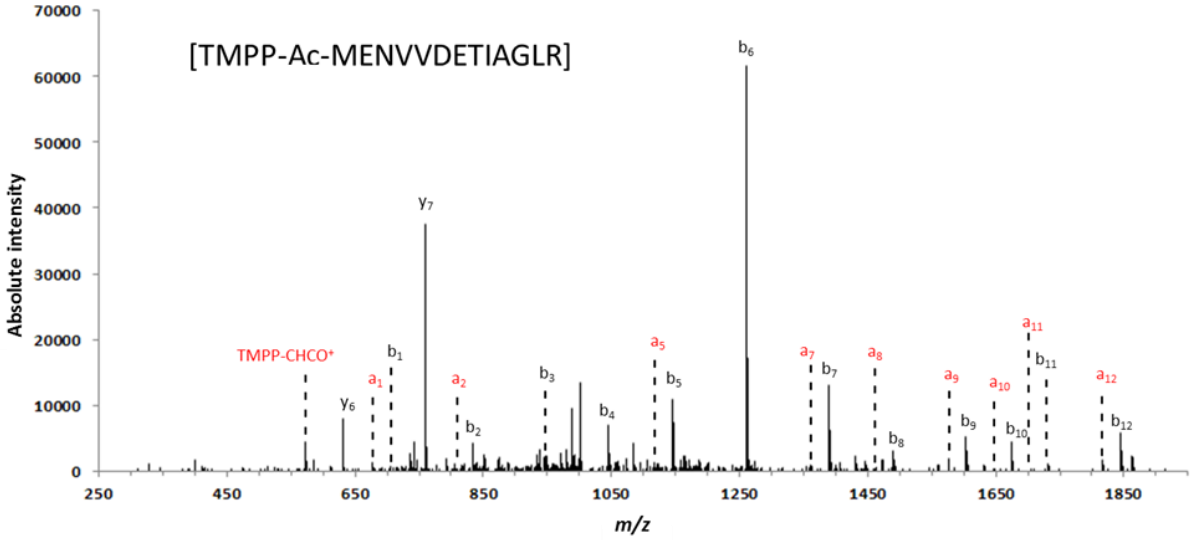
    >gi|56695141:239589-239693 Ruegeria pomeroyi DSS-3, complete genome
     GACCCAGCGCCGGGGATCGGTCGGCGCGCCCTA * GAGGACGATGTCCTGTTACCTCATACAGCGGCGGCCATGCCCGCC---CATGCAGGGGCCAAAATGATG---GCCCAAGGCACTTATACCAGCGAACAGCTGCTGGACCGC
1                                                                             M  P  A  -  H  A  G  A  K  M  M  -  A  Q  G  T  Y  T  S  E  Q  L  L  D  R

2                                                    M  L  P  H  T  A  A  A  M  P  A  -  H  A  G  A  K  M  M  -  A  Q  G  T  Y  T  S  E  Q  L  L  D  R

    >gi|84515773:121203-121346 Loktanella vestfoldensis SKA53, complete genome
     ATGCCCTGCGCCGAGGCTTTGTCGGCGCCAACCCAGGAGGACAATGCACTGTCTCTTTCCCAATCGGCTGCACCGGCGGCCACCATCGGAGCCTATGCCATG---CAGGCTCTGAATATCGTGAATAGCGAAACCATTCTGGCGGCT
1     M  P  C  A  E  A  L  S  A  P  T  Q  E  D  N  A  L  S  L  S  Q  S  A  A  P  A  A  T  I  G  A  Y  A  M  -  Q  A  L  N  I  V  N  S  E  T  I  L  A  A

2                                                    M  S  L  S  Q  S  A  A  P  A  A  T  I  G  A  Y  A  M  -  Q  A  L  N  I  V  N  S  E  T  I  L  A  A
```

Figure 5



NC008209Rdesnitrificans_-1_331094@330567 ***Roseobacter Denitrificans*****, complete genome, (reverse complement 330567-331094)**

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                   M  E  N  V  V  D  E  T  I  A  G  L  R  S  V  L  S  A  Q  N  N  A

GAGTTGGCTAGAAAATTGGGTATTGATCAAAGCACAATATCGTCTTGGCGTTCGCGGGGTCGTGTACCAAACCGATTTGTTCAA
 E  L  A  R  K  L  G  I  D  Q  S  T  I  S  S  W  R  S  R  G  R  V  P  N  R  F  V  Q

ATGCTTGAGCCTCCAAAAGAGGGCGTGTCAAAGAACGCTACACAAAGTTGGCCAGAACTTGAGGAGCGGGGCCAGTCTATTGCT
 M  L  E  P  P  K  E  G  V  S  K  N  A  T  Q  S  W  P  E  L  E  E  R  G  Q  S  I  A

TTAGTGCGCTTCACAATTCTTAGACAAAAAATAGCTTCCAGCGGTGACGTAGATACAGCGCTACCAGTCTTTTTGGACTTGCTG
 L  V  R  F  T  I  L  R  Q  K  I  A  S  S  G  D  V  D  T  A  L  P  V  F  L  D  L  L

CCGTTTTGGTTGGTGATGCATCGCGCGGTGCATGAGCTAAGGCTAAAAATGGAGGCGTTGAAGGTAGACTTGAAAACTGCGCAG
 P  F  W  L  V  M  H  R  A  V  H  E  L  R  L  K  M  E  A  L  K  V  D  L  K  T  A  Q

GCCCTTTTGATGCAAGAGGATTTGCGTGATCCACAGGCTACTCTGGATAGGGTGGCCAACCAGTTGGAAGAAGACCTGAAAGAC
 A  L  L  M  Q  E  D  L  R  D  P  Q  A  T  L  D  R  V  A  N  Q  L  E  E  D  L  K  D

AATCCACATCTCAAAGAATGGAAGTAG
 N  P  H  L  K  E  W  K  *
```

Figure 6