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Anaerobic growth promotes synthesis of colonization factors encoded at the *Vibrio* pathogenicity island in *Vibrio cholerae* El Tor

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Abstract

Pathogenesis of the facultative anaerobe *Vibrio cholerae* takes place at the gut under low oxygen concentrations. To identify proteins which change their expression level in response to oxygen availability, proteomes of *V. cholerae* El Tor C7258 grown in aerobiosis, microaerobiosis and anaerobiosis were compared by two-dimensional electrophoresis. Twenty-six differentially expressed proteins were identified which are involved in several processes including iron acquisition, alanine metabolism, purine synthesis, energy metabolism and stress response. Moreover, two proteins implicated in exopolysaccharide synthesis and biofilm formation were produced at higher levels under microaerobiosis and anaerobiosis, which suggests a role of oxygen deprivation in biofilm development in *V. cholerae*. In addition, six proteins encoded at the *Vibrio* pathogenicity island attained the highest expression levels under anaerobiosis, and five of them are required for colonization: three correspond to toxin-coregulated pilus biogenesis components, one to soluble colonization factor TcpF and one to accessory colonization factor A. Thus, anaerobiosis promotes synthesis of colonization factors in *V. cholerae* El Tor, suggesting that it may be a key in vivo signal for early stages of the pathogenic process of *V. cholerae*.
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1. Introduction

Vibrio cholerae is the etiological agent of the severe diarrheal disease cholera, which is caused by colonization of the human small bowel by strains of the O1 and O139 serogroups. To cause disease, *V. cholerae* must assemble colonization factor toxin-coregulated pilus (TCP) and secrete cholera toxin (CT). Expression of genes encoding TCP, CT and other virulence factors is coordinately regulated by the ToxR regulon in response to external specific stimuli in vitro [18,22]; however, the signals modulating the ToxR regulon in vivo are not well known. *V. cholerae* may sense, in the intestine, signals like bile, pH, lack of oxygen and nutrient availability. The adaptation of *V. cholerae* to these physiologically relevant conditions is of interest.

Sequencing of the *V. cholerae* N16961 genome [9] has led to elucidation of global expression patterns of this organism at transcriptional and proteomic levels. According to DNA microarrays, *V. cholerae* infecting rabbit ileal loops [32] or shed in the stools of cholera patients [1,24] experiences anaerobiosis, iron limitation and nutrient deprivation. In

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addition, an anaerobically grown *V. cholerae* classical strain shows uncoupled expression of CT and TCP [17], a pattern resembling that occurring in the suckling mouse intestine at early stages of infection [20].

Thus, we were interested in studying the response of *V. cholerae* to oxygen deprivation on a proteomic scale. Changes in the proteome of a *V. cholerae* El Tor strain grown under anaerobiosis were analyzed by Kan et al. [14]. Bacteria were cultured in Luria–Bertani broth in anaerobic jars, in an attempt to approximate the environment of *V. cholerae* during infection [14]. However, the intestinal condition is complex and cannot be completely reproduced by growth in a single culture medium such as Luria–Bertani. Moreover, cells studied by Kan et al. were cultured to stationary phase in which *V. cholerae* does not divide actively, as has been suggested to occur in vivo [1,19]. Thus, among proteins which are more abundant under anaerobiosis, Kan et al.'s analysis did not identify any that were related to pathogenesis. Accordingly, further studies on the proteomes of *V. cholerae* growing under different oxygen concentrations, culture media and growth phases may shed new light on the regulation of virulence factor expression.

Therefore, we compared proteomes of *V. cholerae* El Tor C7258 grown to exponential phase under aerobiosis and anaerobiosis in syncase medium, attempting to mimic the neutral pH, amino-acid-rich environment and carbon source found by *V. cholerae* in the small intestine [6]. In addition, and since the small bowel is recognized to be microaerobic at its first portion and gradually becomes anaerobic as it approaches the large intestine [27], the proteome of microaerobically grown vibrios was also analyzed.

2. Materials and methods

2.1. Bacterial strain and culture conditions

V. cholerae C7258 (O1, El Tor, Ogawa; Peru, 1991) was grown overnight in syncase (Na_2HPO_4 and K_2HPO_4, 5 g l^{-1}; NH_4Cl, 1.18 g l^{-1}; casein hydrolysate, 10 g l^{-1}; pH 7.5 supplemented with glucose, 4 g l^{-1}; Na_2SO_4, 626 μM; $MgCl_2 \cdot 6H_2O$, 206 μM; $MnCl_2 \cdot 4H_2O$, 20 μM; and $FeCl_3$, 18 μM) [6]. The overnight culture was diluted 1:100 in the same medium and was grown at 220 rpm and 37 °C to optical density (OD) at 600 nm of ∼0.2. Two milliliters of this culture were inoculated in parallel in 200 ml of syncase, previously stirred overnight under an aerobic, microaerobic or anaerobic atmosphere. Microaerobic and anaerobic environments were generated with Campy*Gen*™ and Anaero*Gen*™ sachets, respectively, in sealed jars (Oxoid). The cultures were shaken under each atmosphere at 220 rpm and 37 °C to an OD_{600} of ∼0.5.

2.2. Sample preparation, gel electrophoresis and image analysis

Bacterial pellets (∼10^{10} cells) were washed twice with buffered sucrose (270 mM sucrose, 1.3 mM Na_2HPO_4, 1 mM $MgCl_2$; pH 7.4) and incubated for 10 min in a lysis solution containing 7 M urea, 2 M thiourea, 2% CHAPS, 0.5% ASB-14, 15% glycerol and 1% DTT. Nucleic acids were removed by centrifugation at 60,000 × *g* for 3 h in the presence of 2% ampholines, and supernatants were delipidated by extraction with ethyl ether.

Proteins were loaded onto an immobilized pH gradient strip (BioRad) by in-gel rehydration. About 150 μg and 500 μg of proteins were used for each analytical and preparative gel, respectively. Two-dimensional gel electrophoresis (2-DE) was performed in a Multiphor II electrophoresis system combining isoelectric focusing in immobilized pH gradient strip (pH 4–7, focusing at 75 kV/h) and vertical SDS–PAGE (18 × 20 cm, 16% of acrylamide). Silver staining of analytical gels was done according to Heukeshoven and Dernick [10], while preparative gels were silver-stained with a mass spectrometry (MS)-compatible procedure [13].

Gel images were recorded with a ScanJet 6300C at a resolution of 600 dpi and analyzed using Melanie III software (GeneBio). For each gel, automatic detection, edition, area and volume calculation of spots was done twice and values were compared to evaluate consistency of the data. For each condition, gel reproducibility was evaluated by pair reports and scatter plot analysis. Heuristic clustering was used for automatic classification. Detection of changes in protein expression under aerobic, microaerobic and anaerobic conditions was first done by visual inspection of the series of gels and confirmed by automatic analysis. The percent of the volume (% volume) each spot represents in the total volume of all spots in each gel was used to compare protein expression across conditions. Histograms on groups based on % volume of spots were recorded using the central tendency as the mean value and the mean square deviation as the dispersion value. Spots which consistently changed their intensities more than twofold (determined from the mean) between aerobic and anaerobic conditions were considered for identification by MS.

2.3. In-gel digestion, MS analysis and protein identification

In-gel digestion of spots and recovery of proteolytic peptides were performed as previously described [8].

Low-energy electrospray ionization–MS spectra were acquired using a hybrid quadrupole orthogonal acceleration tandem mass spectrometer QTof-2™ (Waters) fitted with a Z-spray nanoflow electrospray ion source. Measurement conditions were similar to a previous report [8].

Proteins were identified by peptide mass fingerprinting (PMF) using ProFound (http://prowl.rockefeller.edu/prowl-cgi/profound.exe). The electrospray ionization–MS spectra were deconvoluted using the MaxEnt 3.0 software (Waters, Milford, USA) and peak lists containing the monoisotopic signals were created after excluding signals originating by blank digestion and from known keratin peaks. One missed cleavage site for trypsin was considered for the matching peptides within an error below 0.05 Da. MW for proteins searched in the NCBI nr (28/09/03) database was restricted to those values approximately detected in 2-DE gels (6 kDa ≤ protein mass ≤ 150 kDa) and

p*I* ($3 \leq pI \leq 8$). Carbamidomethylcysteine and methionine sulfoxide were considered in all database searches as fixed and variable modifications, respectively, for all peptides containing these amino acids. Identifications by PMF were considered reliable when sequence coverage was higher than 20%, more than five matching peptides were found and expectation values were significantly below 10^{-4}. Identifications by Profound were confirmed by sequencing at least one or two matching peptides.

The raw electrospray ionization-tandem MS spectra were deconvoluted by MaxEnt 3.0, exported as a DTA format and loaded into MASCOT software to complete sequence identification (http://www.matrixscience.com/search_form_select.html). Identifications were considered positive when the score indicated significant homology between the experimental and expected electrospray ionization–tandem MS spectra ($p < 0.05$), when more than four consecutive y_n'' ions were assigned to intense signals and results provided by MASCOT and PepSea were coincident, or when two or more sequenced peptides identified the same protein. With the option of MSMS ion search provided by MASCOT, parameters used were as follows: (1) trypsin with one missed cleavage site, (2) carbamidomethylcysteine and methionine sulfoxide plus Asn deamidation as fixed and variable modifications, respectively, (3) peptide and MSMS tolerances of 1.2 and 0.6 Da, respectively, (4) no taxonomic restrictions, and (5) use of the NCBI nr database (updated by the MASCOT online version). Similar parameters were introduced in the case of PepSea software. Electrospray ionization–tandem MS spectra were manually interpreted to extract reliable sequence tags (usually 3–5 amino acids) using intense y_n'' ions located in the less complicated region of the spectra and confirmed by their respective complementary b_n ions whenever possible. This information was loaded either onto PepSea software or onto the sequence query option provided by MASCOT to perform protein identification. In MASCOT as well as in PepSea, a non-tolerant error database search was defined to identify peptides based on their MSMS spectra. For this option, once the peptide was identified, the entire MSMS spectrum was carefully examined to critically evaluate the assignment by searching for the remaining backbone ions so as to support or reject identification and to avoid having intense signals which remained without assignment and hence false identifications. The identifications performed using this procedure were coincident with automatic interpretation of MSMS spectra with significant scores. Most identifications were supported by coincident identifications of two or more peptides. No taxonomic restrictions were introduced for any searches, and therefore, when a *V. cholerae* protein was identified among the best ranked sequences, it was considered to be an indication of positive identification.

2.4. Immunoassays

Cholera toxin in culture supernatants was determined by a standard GM_1 enzyme-linked immunosorbent assay and expression of TcpA was analyzed by Western blotting as previously described [4].

3. Results

3.1. Global changes in the V. cholerae C7258 proteome in response to oxygen availability

Protein samples from three independent cultures of *V. cholerae* C7258 grown in syncase broth under aerobic, microaerobic and anaerobic conditions were analyzed by 2-DE. Automatic heuristic clustering performed on nine scanned gel images correctly grouped them into three classes, corresponding to the analyzed conditions. Image analysis led to detection of spots corresponding to differentially expressed proteins. The differentially expressed proteins in microaerobiosis coincided with those detected in aerobiosis and anaerobiosis, but their expression levels were in most cases intermediate between those observed in aerobiosis and anaerobiosis. Accordingly, the differentially expressed spots under these two conditions were considered for identification by MS. Twenty-six proteins with expression levels which varied by more than twofold between aerobic and anaerobic conditions were identified (Fig. 1, Table 1; supplementary table). Eight of them were less frequently expressed under hypoxic conditions (micro- and anaerobiosis) than in aerobiosis, showing higher expression levels in microaerobiosis than in anaerobiosis (Table 1). Conversely, among the 26 identified proteins, 18 were more strongly expressed in hypoxic cultures than in aerobic cultures (Table 1). Only 2 of these 18 proteins showed higher amounts in microaerobiosis (spots 353 and 916; Table 1, Fig. 1).

3.2. Identified proteins produced at lower levels under hypoxic growth of V. cholerae C7258

Tricarboxylic acid (TCA) cycle succinyl-CoA synthetase (SucD), catabolic alanine dehydrogenase (Ald) and the biosynthetic enzymes alanine racemase (Alr2) and the catalytic subunit of phosphoribosylaminoimidazole carboxylase (PurE) (Spots 578, 283, 375 and 817, respectively; Table 1) were less strongly expressed under hypoxic conditions.

The periplasmic components of three ferric iron-ATP binding cassette transporters, products of VC0202, VC0608 and VCA0227 and putative iron-regulated protein IrpA (spots 372, 462, 562 and 236, respectively; Table 1), were also less strongly expressed in hypoxic cultures. The products of VC0202 (FhuD) and VCA0227 (VctP) bind and transport ferrichrome- and vibriobactin/enterobactin-chelated ferric ions, respectively, and the product of VC0608 (FbpA) transports unchelated ferric ions [31]. The function of IrpA, on the other hand, has not been experimentally determined, but its co-expression with other proteins related to iron transport suggests its involvement in iron uptake.

3.3. Identified proteins more strongly expressed in hypoxic cultures of V. cholerae C7258

Glyceraldehyde-3-phosphate dehydrogenase (GapA-1), formate acetyltransferase (PflB), alcohol/acetaldehyde dehydrogenase (AdhE), aspartate ammonia-lyase (AspA) and the

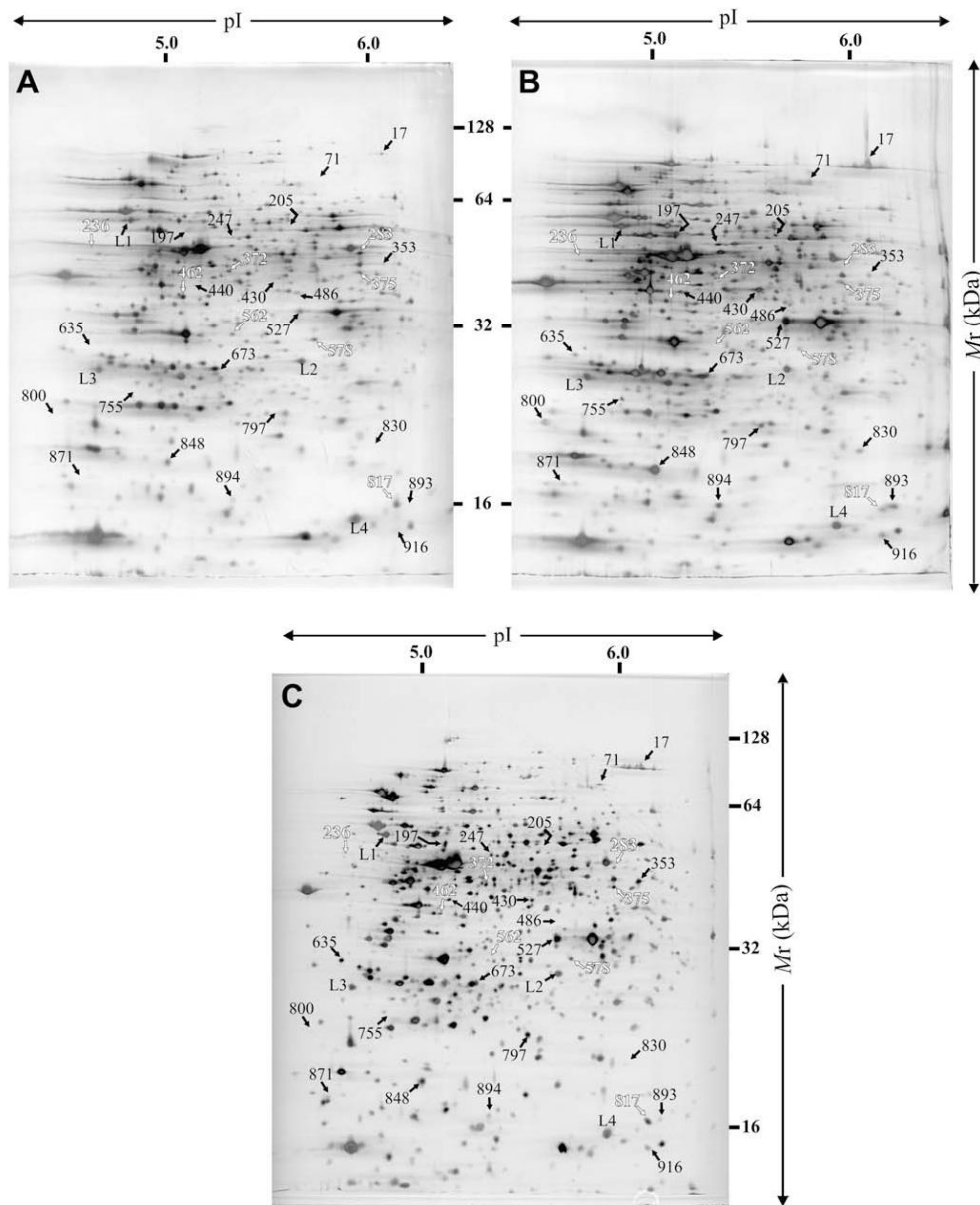

Fig. 1. 2-DE maps of proteins from *V. cholerae* C7258 grown under aerobiosis (A), anaerobiosis (B) and microaerobiosis (C). The numbers indicate spots identified by mass spectrometry as shown in Table 1. L1, L2, L3 and L4 designate landmarks and correspond to beta subunit of ATP synthase F1 (50.5 kDa, p*I* = 4.8), uridylate kinase (26.2 kDa, p*I* = 5.7), heat shock protein GrpE (22.7 kDa, p*I* = 4.6) and ribosomal protein S6 (14.2 kDa, p*I* = 6.0), respectively. Open and solid arrows mark protein spots whose amounts decreased and increased, respectively, in anaerobically grown cells.

iron–sulfur protein of the fumarate reductase complex (FrdB) (spots 527, 71, 17, 197 and 673, respectively; Table 1, Fig. 2), all related to energy metabolism, were produced at higher levels under hypoxic growth. A putative formate transporter (Spot 797; Table 1, Fig. 2), probably related to anaerobic metabolism, was also increased under these conditions. In microaerobiosis, these proteins attained almost the same expression level as under anaerobiosis (Table 1).

TCP biosynthesis proteins C, Q, S and F, accessory colonization factor A (AcfA) and probable thioredoxin peroxidase (TagD-former ToxR-activated gene D) (spots 205/247, 800/871, 893, 430/486, 755 and 848, respectively; Table 1, Fig. 3A) also increased their amounts under hypoxic growth, showing the highest expression levels under anaerobiosis. These proteins are encoded at the *Vibrio* pathogenicity island [15] and five of them are required for intestinal colonization: TcpC, TcpQ and TcpS correspond to the toxin-coregulated pilus biogenesis apparatus [21], TcpF is a soluble colonization factor [16] and AcfA is part of a less well characterized colonization factor [26]. Thus, anaerobic growth promoted synthesis of colonization factors in the El Tor *V. cholerae* C7258.

Only four of the 12 *tcp* biogenesis operon-encoded proteins were detected at higher levels under anaerobiosis (Fig. 3B). This apparently reflects 2-DE limitations for detecting TcpB (minoritary), TcpD and TcpR (cytoplasmic-membrane-located) and TcpA, TcpT, TcpE and TcpJ, which electrofocus

Table 1
Identified differentially expressed proteins from *V. cholerae* C7258 by MS.

Spot[a]	Protein name[b]	MM (kDa)/p*I*[c]	Peptides sequenced[d]	Score PMF (expectat.)/% coverage[e]	Induction ratio[f] A/N	A/M	M/N	Biological role[g]
Proteins less strongly expressed in anaerobiosis and microaerobiosis								
236	Iron-regulated protein A, putative	43.2/4.6–**42/4.6**	1	–	N–	1.28	N–	Transport and binding proteins
283	Alanine dehydrogenase[h,i,k]	39.8/5.9*	2	–	N–	3.33	N–	Energy metabolism
372	Iron(III) ABC transporter, periplasm. iron-comp. binding protein	31.2/5.3–**38/5.3**	1	–	4.23	2.11	2.01	Transport and binding proteins
375	Alanine racemase	41.7/5.9–**38/5.4**	2	–	2.22	1.57	1.41	Cell envelope
462	Iron(III) ABC transporter, periplasm. iron-comp. binding protein[i]	35.4/5.1–**35/5.1**	1	–	9.91	3.57	2.77	Transport and binding proteins
562	Iron(III) ABC transporter, periplasm. iron-comp. binding protein	31.4/5.4–**31/5.3**	2	–	N–	3.49	N–	Transport and binding proteins
578	Succinyl-CoA synthetase, alpha subunit[i,k]	29.9/5.8*	2	–	3.60	1.55	2.32	Energy metabolism
817	Phosphoribosylaminoimidazole carboxylase, catalytic subunit	16.6/6.2*	3	–	2.18	1.17	1.86	Purines
Proteins more strongly expressed in anaerobiosis and microaerobiosis								
17	Alcohol/acetaldehyde dehydrogenase	96.2/6.1*	2	2.3×10^{-5}/28%	0.19	0.28	0.68	Energy metabolism
71	Formate acetyltransferase	87.8/6.0–**79/5.8**	2	0.13 /14%	A–	0	0.90	Energy metabolism
197	Aspartate ammonia-lyase	52.8/5.1*	3	9.4×10^{-5}/32%	0.12	0.14	0.91	Energy metabolism
205	TCP biosynthesis outer membrane protein C	52.4/5.4–**52/5.6**	3	–	A–	0	0	Cellular processes
247		52.4/5.4–**49/5.3**	2	9.8×10^{-7}/37%	A–	0	0.50	
353	UDP-*N*-acetyl-D-mannosaminuronic acid dehydrogenase	44.5/6.0–**43/6.1**	3	–	0.36	0.11	3.31	Cell envelope
430	TCP biosynthesis outer membrane protein F	35.8/5.5–**39/5.5**	2	2.4×10^{-9}/57%	0.09	0.24	0.36	Cellular processes
486		35.8/5.5–**37/5.7**	1	–	A–	0	0	
440	Peptidase, M20A family[j]	39.3/5.2*	3	0.89 /20%	0.24	0.29	0.86	Protein fate/protein synthesis
527	Glyceraldehyde 3-phosphate dehydrogenase[i,j]	35.3/5.8–**34/5.6**	3	0.028/35%	0.09	0.074	1.25	Energy metabolism
635	Hypothetical protein	26.4/4.6–**29/4.6**	4	–	A–	0	2.12	Hypothetical protein
673	Fumarate reductase iron-sulfur protein	28.0/5.3*	5	–	A–	0	1.16	Energy metabolism
755	Accessory colonization factor A	22.8/4.9–**24/4.8**	2	–	A–	0	0	Cellular processes
797	Formate transporter 1, putative	52.3/6.5–**23/5.5**	3	0.33 /16%	A–	0	1.06	Transport and binding proteins
800	TCP biosynthesis protein Q	15.0/4.4–**22/4.4**	1	–	A–	0	0.16	Cellular processes
871		15.0/4.4–**8/4.5**	1	–	A–	0	0.24	
830	Hypothetical protein	16.5/6.0–**21/6.0**	2	–	A–	0	0.21	Hypothetical protein
848	Probable thioredoxin Peroxidase	17.9/5.0–**19/5.1**	6	–	0.52	0.90	0.58	Cellular processes
893	TCP biosynthesis protein S	15.1/6.2–**17/6.3**	3	–	A–	0	0.21	Cellular processes
894	16 kDa heat shock protein A[j,k]	16.8/5.3*	3	2×10^{-3}/54%	0.21	0.74	0.29	Protein fate/protein synthesis
916	Conserved hypothetical protein[k]	14.0/6.0–**13/6.2**	1	2.2×10^{-3}/49%	A–	0	0.75	Hypothetical protein

[a] Number assigned by Melanie software.
[b] Protein name according to The Institute for Genomic Research (http://cmr.tigr.org/cgi-bin/CMR/GenomePage3.cgi?org=gvc).
[c] Theoretical values for mature proteins (according to analysis by SignalP 3.0, http://www.cbs.dtu.dk/services/SignalP/) calculated using Expasy tool Compute p*I*/Mw (http://www.expasy.org/tools/pi-tool.html). Bold typed numbers, experimental values of MM/p*I*; *MM/p*I* of proteins used for gel calibration.
[d] Peptides sequenced by MSMS. *m/z* calc., *m/z* exp. z and Mascot score of peptide identification are included in the supplementary table.
[e] –: Identification by PMF was not successful. Matching peaks and total peaks are provided in supplementary table.
[f] Ratio between the values (% volume) of each spot in: A/N, aerobiosis and anaerobiosis; A/M, aerobiosis and microaerobiosis; M/N, microaerobiosis and anaerobiosis; A– or N–: spot not detected (volume spot <0.02) in the three aerobic or anaerobic gels, respectively.
[g] Biological role according to The Institute for Genomic Research *V. cholerae* genome homepage (http://www.tigr.org/tigr-scripts/CMR2/gen_table.spl?db=gvc).
[h] Protein identified in a *V. cholerae* classical strain grown aerobically in a minimal medium [11].
[i] Proteins identified in *V. cholerae* El Tor strains grown aerobically in minimal or Luria–Bertani (LB) media [2].
[j] Proteins more abundant in a *V. cholerae* El Tor strain grown anaerobically in LB [14].
[k] Proteins with increased levels in a *phoB* mutant grown aerobically, under inorganic phosphorus starvation, compared to parental strain *V. cholerae* 569B [29].

out of the p*I* range achieved in the study (Fig. 3B). Accordingly, the proteomic samples were examined by Western blot for the presence of TcpA, the first protein encoded at the *tcp* biogenesis operon. TcpA was present in microaerobically and anaerobically grown but not in aerobically grown C7258 (Fig. 3C), indicating that although it was not detected in anaerobic and microaerobic 2-DE gels, it was being synthesized under these conditions.

To determine whether increased accumulation of TCP proteins was coupled to high levels of CT, as occurs in AKI growth (a condition allowing co-expression of CT and TCP in El Tor strains) [23], supernatants from aerobic and anaerobic

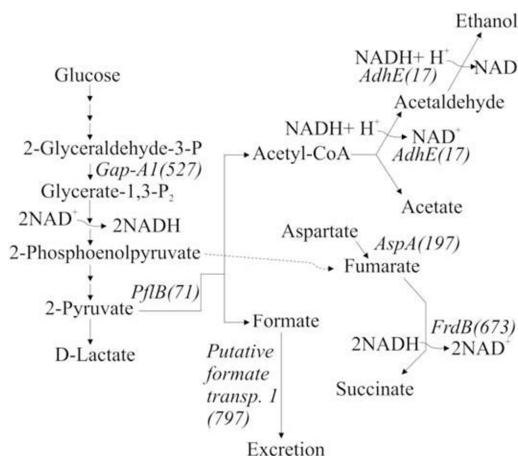

Fig. 2. View of glycolytic and pyruvate dissimilation pathways used by bacteria during anaerobic growth. Proteins whose accumulation increased in *V. cholerae* during hypoxic growth are indicated. Gap-A1, glyceraldehyde 3-phosphate dehydrogenase; PflB, formate acetyltransferase; AdhE, alcohol/acetaldehyde dehydrogenase; AspA, aspartate ammonia-lyase; FrdB, iron–sulfur protein of fumarate reductase. The number of the corresponding spot is shown in parentheses.

cultures were evaluated in a GM_1 enzyme-linked immunosorbent assay. Remarkably, the higher levels of TCP proteins achieved under anaerobiosis were coupled with only a slight increase in CT production (aerobiosis <1 ng ml^{-1} OD_{600}^{-1} and anaerobiosis ~8 ng ml^{-1} OD_{600}^{-1}). Thus, in El Tor strain C7258, anaerobic growth under a traditionally repressive condition (syncase medium) stimulated synthesis of TCP operon-encoded proteins to high levels, but CT was poorly induced; thus, coordinate expression of TCP and CT reported under AKI conditions (C7258 produces up to ~150 ng ml^{-1} OD_{600}^{-1} of CT) was not observed in anaerobic syncase growth.

Moreover, UDP-*N*-acetyl-D-mannosaminuronic acid dehydrogenase (VpsB) and the product of VC0928 (RbmA), formerly a hypothetical protein (spots 353 and 635, respectively; Table 1), were more strongly expressed under hypoxic conditions, showing the highest levels in microaerobiosis (Fig. 1, Table 1). VpsB is required for biosynthesis of the *Vibrio* exopolysaccharide [33] and RbmA for rugose colony formation and biofilm structure integrity [7]. *Vibrio* polysaccharide is also essential for the appearance of the colony morphology and biofilm development [33].

Finally, 16 kDa heat shock protein A (HspA) and a peptidase of the M20A family were also better expressed in hypoxic cultures (Spots 440 and 894, respectively; Table 1), which coincides with results of Kan et al. [12] and suggests that these two products play a role during adaptation to growth under these conditions, even in different culture media.

3.4. Identified hypothetical proteins present in larger amounts under hypoxic growth of V. cholerae *C7258*

The conserved hypothetical protein encoded by VC2216, which was induced in response to phosphate starvation in a *V. cholerae pho*B mutant [29], and hypothetical proteins coded by VCA0260 and VCA0261 were more strongly expressed under hypoxic conditions (spots 916 and 830, respectively; Table 1). The experimental values for MM and p*I* of most proteins identified in this report were reasonably close to their predicted values (Table 1, MM/p*I*), including those for products of VC2216. However, this was not the case for hypothetical proteins encoded by VCA0260 and VCA0261.

The electrospray ionization-MS spectrum of tryptic peptides of spot 830 (not shown) displayed two signals at *m*/*z* 610.63 (Fig. 4A) and 628.34 (Fig. 4B) whose sequences (Fig. 4C and D) corresponded to products of VCA0260 and VCA0261, overlapped genes on chromosome II of N16961 [9]. The experimental p*I* of spot 830 (6.05) was close to values predicted for products of VCA0260 (6.5) and VCA0261 (6.0), but its experimental MM (21 kDa) was higher than those predicted for products of VCA0260 (7.3 kDa) and VCA0261 (11.9 kDa). BLASTP sequence alignment of proteins encoded by VCA0260 and VCA0261 showed high similarity to the C- and N-terminus, respectively, of a single 21 kDa protein in *Vibrio vulnificus* YJ016 (VVA1319) and CMCP6 (VV20853) and in *Vibrio parahaemolyticus* RIMD 2210633 (VPA0479). These elements suggest that VCA0260 and VCA0261 may constitute a single gene coding for a 21 kDa protein in C7258. Additional experiments are in progress to test this hypothesis.

4. Discussion

The proteome of *V. cholerae* El Tor C7258 grown in glucose-containing, iron-sufficient (18 μM) syncase broth under aerobiosis, microaerobiosis and anaerobiosis is analyzed and reported here for the first time. C7258 responded to micro- and anaerobiosis, causing changes in cellular processes such as metabolism (10 proteins), iron uptake (4 proteins), pathogenesis (6 proteins), surface polysaccharide synthesis and biofilm maturation (2 proteins), protein fate (2 proteins) and hypothetical proteins with unknown function (2 proteins) (Table 1). Although the spectrum of modified processes is wide, the 26 changes identified were far smaller than the hundreds of genes found to be regulated by oxygen in *Escherichia coli* [31]. Thus, further analyses of the *V. cholerae* proteome using more sensitive methods might shed additional light on modifications in expression levels of less abundant proteins.

4.1. Most changes in V. cholerae *C7258 in micro- and anaerobiosis account for metabolic shift*

Our observation that the TCA cycle SucD enzyme, along with an earlier report that the flavoprotein subunit of succinate dehydrogenase [14], are less abundant proteins under hypoxic conditions suggest inhibition of the cycle under these conditions, and coincide with reports for *E. coli* stating that most genes of the TCA cycle are anaerobically repressed [3]. Increases in FrdB and AspA expression levels in C7258 under hypoxic conditions (Fig. 2), in addition, suggest operation of a branched TCA cycle as in *E. coli* [3]. When a bifurcated TCA cycle is functioning and no final electron acceptor is

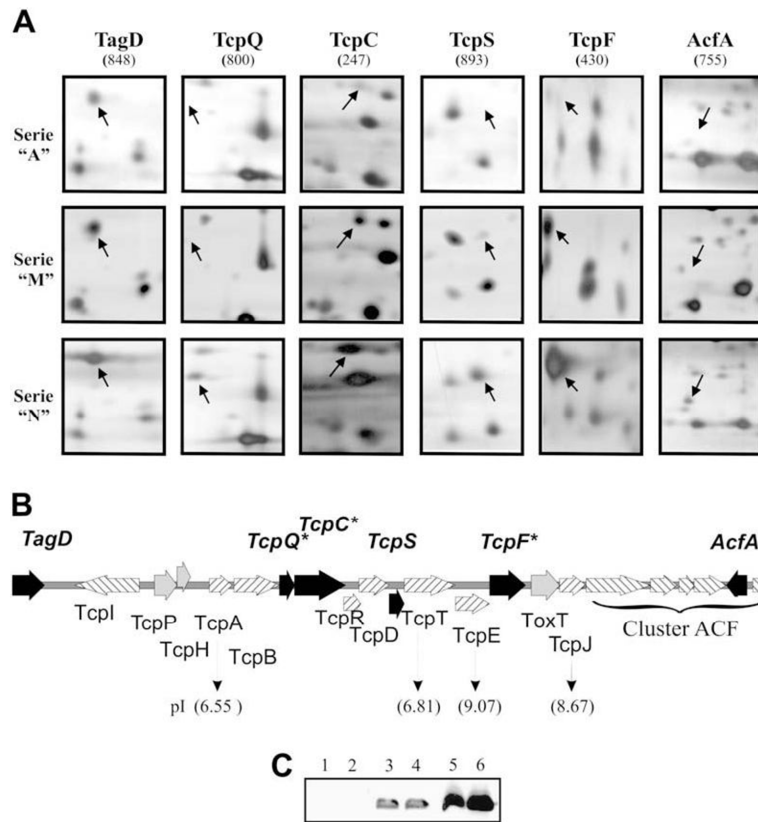

Fig. 3. Pathogenesis-related proteins showing differential expression. (A) 2-DE gel regions containing spots of interest from aerobic (series "A"), microaerobic (series "M") and anaerobic gels (series "N"). (B) Gene organization of the TCP cluster and adjacent genes within the *Vibrio* pathogenicity island. Solid arrows: genes encoding proteins identified by MS; gray arrows: genes encoding regulatory proteins; left cross-hatching: genes encoding proteins not implicated in biogenesis of TCP; right cross-hatching: genes encoding proteins directly involved in TCP biosynthesis. Bold type: proteins identified by MS; *: proteins showing two different electrophoretic mobilities. Theoretical p*I* values of mature and precursor forms (in parentheses) were calculated using Expasy tool Compute p*I*/MW (http://www.expasy.org/tools/pi_tool.html) of the unidentified proteins directly involved in TCP biogenesis. (C) Western blot for TcpA of C7258 cells grown under aerobiosis (lanes 1 and 2), microaerobiosis (lanes 3 and 4) and anaerobiosis (lanes 5 and 6).

added, the glycolytic pathway and substrate phosphorylation play a central role in fulfilling the cell's energy demands. Accordingly, the glycolytic enzyme GapA-1 was found to be 10-fold more expressed under hypoxic conditions, coinciding with findings of Kan et al. [14]. Moreover, higher amounts of PflB and AdhE under hypoxic conditions indicate the occurrence of fermentation in C7258 cultures (Fig. 2). The putative formate transporter identified in this work might excrete formate produced by PflB. Interestingly, proteins associated with anaerobic metabolism attained almost the same expression levels in microaerobiosis as in anaerobiosis (Table1). Coincidently, a switch from aerobic to anaerobic respiration in *E. coli* occurs under microaerobiosis [33], and microaerobically grown *E. coli* cells have active metabolic pathways characteristic of anaerobic metabolism [7].

In addition, *V. cholerae* seems to readjust metabolic pathways beyond those of the central energy metabolism in response to oxygen depletion. Thus, Ald, Alr2 and PurE were less strongly expressed in hypoxic cultures. Lesser amounts of an enzyme of the de novo purine biosynthesis pathway (PurE) in hypoxic cultures coincides with the previous report in *V. cholerae* [14] and might represent a specific response to oxygen limitation, shutting down an energy-expensive pathway. Additional studies are necessary to determine the cause of modulation of Ald and Alr2 expression by oxygen.

4.2. *An aerobic environment increases expression of ferric iron transporters in* V. cholerae *C7258 grown in iron-sufficient broth*

Detection of higher production of IrpA, VctP, FbpA and FhuD in C7258 grown under aerobiosis coincides with identification of the periplasmic component of other ferric iron-ATP binding cassette transporter as being more abundant in aerobiosis [14]. These findings suggest that, under this condition, *V. cholerae* induces synthesis of ferric iron transporters. Genes encoding IrpA, VctP, FbpA and FhuD are regulated by Fur and iron [25,28]. Aerobic increases in ferric iron transporters reflect the presence of iron in the non-bioavailable Fe^{3+} redox state in cultures supplemented with 18 μM $FeCl_3$. Ferric hydroxide complexes formed in oxygenated atmospheres likely lower iron bioavailability under these conditions [31]. Accordingly, the expression level of all these proteins under microaerobiosis was lower than under aerobiosis (Table 1). In anaerobiosis, however, iron is found soluble as ferrous ions, which are transported by the FeoABC system [31].

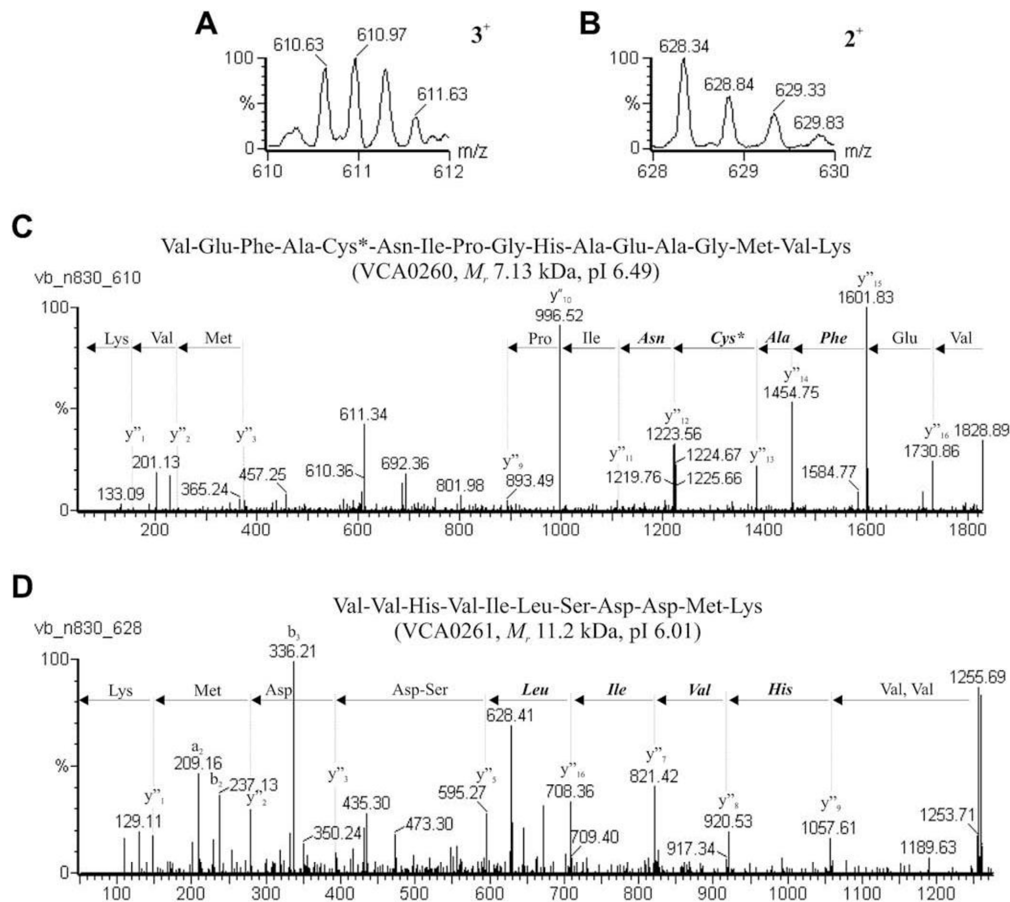

Fig. 4. Expanded regions of electrospray ionization–MS spectrum corresponding to: (A, B) doubly-charged (*m/z* 610.63) and triply-charged (*m/z* 628.34) tryptic peptides of spot 830; (C, D) electrospray ionization-tandem MS spectra of previously mentioned peptides.

4.3. *Enhanced pathogenesis-related and* Vibrio *polysaccharide/biofilm maturation-related protein synthesis under hypoxic growth of* V. cholerae *C7258*

The observation of pathogenesis-related proteins TcpC, TcpQ, TcpS, TcpF, AcfA and TagD as being more highly expressed under hypoxic conditions, with the highest amounts in anaerobiosis, is an interesting finding in this study. Anaerobic increases in these proteins were not detected by Kan et al. [12], suggesting that broth composition and culture growth phase influence virulence expression responses of *V. cholerae* to oxygen deprivation. Interestingly, C7258 grown anaerobically to stationary phase did not show increased amounts of TcpA (not shown). Activation of TCP assembly genes has been suggested to be one of the first steps in colonization of the human intestine [19]; thus, anaerobic induction of colonization factors suggests that anaerobiosis may constitute a signal for early induction of these proteins in vivo.

The higher levels of TCP operon-encoded proteins observed in the C7258 El Tor strain with respect to CT levels coincide with uncoupled expression of CT and TCP observed in an anaerobically grown *V. cholerae* classical strain [17], during in vivo growth of vibrios at early stages of infection in an animal model [20] and in humans [19]. Thus, virulence factor expression under anaerobiosis in *V. cholerae* El Tor resembles that occurring in vibrios grown in vivo.

Higher accumulation of VpsB and RbmA may indicate active *Vibrio* polysaccharide production and induction of rugose colony morphology and/or biofilm maturation in response to low oxygen levels. Exopolysaccharide production, biofilm formation and the rugose phenotype are important for the survival of *V. cholerae* in the environment [30]. However, recent reports have suggested that these processes may also play a role during infection. Bile, a component of the human intestine, induces *V. cholerae* biofilm formation, conferring resistance to bile's bactericidal effect [12]. Biofilm development depends on induction of *vps* and VC0928 expression [12]. Moreover, genes *vps* and VC0928 were induced during infection of rabbit ileal loops [32], and recent studies suggest that cholera patients shed high concentrations of *V. cholerae* as in-vivo-formed biofilms [5]. Thus, increased levels of VpsB and RbmA in response to low oxygen levels suggest that this may be a signal for induction of *Vibrio* polysaccharide and biofilm production in vivo.

Gradual changes in expression levels of proteins detected in microaerobic compared to aerobic and anaerobic cultures indicate the existence of oxygen-sensing and responding systems that allow *V. cholerae* adaptation to different oxygen levels, which may be important during its passage through the human intestine during infection. Thus, increased accumulation of VpsB and RbmA in microaerobiosis compared to anaerobiosis contrasts with lower levels of accumulation of

colonization factors under microaerobiosis (Table 1). We hypothesize that this might reflect a difference in the timing of production of *Vibrio* polysaccharide/biofilm and colonization factors in vivo in response to oxygen availability. The microaerobic environment encountered by *V. cholerae* in the stomach and proximal intestine may be favorable to production of *Vibrio* polysaccharide and biofilm, conferring a survival advantage under acidic conditions as well as in the presence of bile, while expression of colonization factors remains low; however, under anaerobic conditions (at the ileum), *Vibrio* polysaccharide production and biofilm formation may diminish in response to low oxygen levels, and colonization factor expression may be induced.

Appendix A. Supplemental material

Supplementary information for this manuscript can be downloaded at doi: 10.1016/j.resmic.2008.10.005.

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