Effect of N-Acetyl-D-Glucosamine on Gene Expression in *Vibrio parahaemolyticus*

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We analyzed the effect of N-acetyl-D-glucosamine (GlcNAc) on gene expression in the marine bacterium *Vibrio parahaemolyticus*. The total number of genes whose expression was induced and repressed genes in the presence of GlcNAc was 81 and 55, respectively. The induced genes encoded a variety of products, including proteins related to energy metabolism (e.g. GlcNAc and chitin utilization), transport, central metabolism and chemotaxis, hypothetical proteins, mannose-sensitive hemagglutinin pilus (MSHA), and a PilA protein, whereas the repressed genes encoded mainly hypothetical proteins. GlcNAc appears to influence directly or indirectly a variety of cellular processes, including energy metabolism, chitin utilization, competence, biofilm formation and pathogenicity. GlcNAc, one of the most abundant aminosugars in the oceans, is used by *V. parahaemolyticus* as an energy source and affects the cellular functioning of this marine bacterium.

Key words: vibrios, *V. parahaemolyticus*, N-acetyl-D-glucosamine, microarrays, genome plasticity

N-acetyl-D-glucosamine (GlcNAc), the building block of the chitin polymer, is one of the most abundant aminosugars in the oceans (11, 20). Marine organisms, particularly zooplankton, produce significant quantities of chitin in the form of carapaces. Chitin is insoluble and made available as a nutrient to fuel the marine microbial food web by means of chitinolytic microorganisms that transform it into its building blocks (4, 18, 38, 45). Chitin and GlcNAc appear to be important sources of carbon and nitrogen for vibrios (2, 3, 22, 24). These bacteria seem to be major players in the cycling of chitin in the marine environment as they produce a variety of potent chitinolytic enzymes (3, 30, 40). GlcNAc uptake and metabolism is mediated by the *nag* regulon which comprises four main genes (*nagABCE*) (6, 9, 19, 20). The transport of GlcNAc into the cell is mediated by a specific PTS (phosphotransferase system) enzyme encoded by *nagE*. This enzyme transforms the aminosugar in GlcNAc-6-P and pyruvate. Within the cell, GlcNAc-6-P is processed by at least six enzymes and the end products are fructose-6-P, NH₃ and acetate. GlcNAc-6-P is transformed in glucosamine-6-P (GlcN-6-P) by N-acetyl-D-glucosamine deacetylase encoded by the *nagA* gene). Subsequently, GlcN-6-P is transformed in fructose-6-P (or used in the biosynthesis of the cell envelope path) by glucosamine-6-P deaminase (isomerase; encoded by the *nagB* gene). Fructose-6-P can be used in glycolysis to generate energy or it can be processed by glucosamine-6-P synthase, again generating GlcN-6-P. In *Vibrio cholerae*, the gene *nagB* is on chromosome II, whereas *nagE*, *nagA* and *nagC* are on chromosome I (14). Some *V. cholerae* strains have a homologous copy of *nagA* in the vibrio pathogenicity island 2 (17).

The expression of *nag* genes in *Escherichia coli* is induced

in the presence of GlcNAc and tightly regulated by the cAMP-CRP (cyclic AMP receptor protein) complex (32, 33, 36, 37). In *E. coli*, NagC is a repressor of *nagABE* (31, 34, 35). It binds to two sites in the intergenic regions between *nagB* and *nagE*. In addition, there is a cAMP-CRP binding site between the two NagC-binding sites. The two binding sites, named boxes E and B, are oriented to control the expression from the promoters of *nagE* and *nagB*, respectively. In the absence of sugars, NagC represses gene expression and activates the expression of *glmUS*. This operon encodes the enzymes that form GlcNAc-6-P and UDP-GlcNAc. In *V. cholerae* the two binding sites for NagC and the cAMP-CRP binding site are in the intergenic regions of *nagE* and *nagAC* (44). The expression of *nagB* is induced in the presence of GlcNAc, but not by glycerol, D-glucosamine, or D-glucose. It appears that the *nagB* promoter is not affected by D-glucose catabolic repression and is induced even in the presence of glucose and GlcNAc. In order to understand the effect of GlcNAc in different cellular processes of vibrios, a comprehensive approach is needed to permit the concomitant examination of different operons and regulons.

Whole genome sequencing has enabled the construction of microarrays which contain all genes of a given bacterium in a single chip. This technology provides a comprehensive view of global bacterial gene expression. A series of striking studies using microassays demonstrated that chitin (but not GlcNAc) induced competence and serogroup conversion in *V. cholerae* (4, 5, 26, 27). A total of 22 induced genes and 51 repressed genes were found in *Vibrio cholerae* grown on GlcNAc (26, 27). The induced genes included those encoding for the *nagEBA*-1 operon (PTS transport system), the GlcNAc-binding protein VCA0811, and the chemotaxis protein VC0449. The repressed genes encoded succinyl-CoA synthase, components of 2-oxoglutarate dehydrogenase, phos-

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phenolpyruvate carboxylase, NadC transporter, D-glucose binding protein of the ABC transporter, and glyceraldehyde-3-P dehydrogenase. Most of the information concerning chitin and GlcNAc utilization and its effect on gene expression is restricted to a few vibrio species, mainly *V. cholerae*. More than one hundred and fifteen vibrio species have been described, yet it is not known if GlcNAc also influences the processes of chemotaxis, adherence, and competence in other vibrios (2, 3, 6, 9, 13, 19, 20). The aim of the present study was to determine the gene expression profile of *Vibrio parahaemolyticus* raised on GlcNAc in different stages of the growth curve by means of a microarray analysis.

Materials and Methods

Bacterial strain and growth conditions

Vibrio parahaemolyticus RIMD2210633 was originally isolated from a patient suffering from diarrhea in the quarantine sector of Kansai International Airport in 1996. This strain harbors the *tdh* gene and belongs to the O3:K6 serogroup. It has been detected in both clinical cases and in the marine environment (41). RIMD2210633 was grown in the minimum medium M9 (Difco™, Sparks, USA) supplemented with 0.5% maltose and 3% NaCl for 8 hours at 28°C prior to the experiments. The experiments comprised two treatments (maltose and maltose+GlcNAc) with 2 replicates each. An inoculum of the bacterial culture (5 mL) was added into an Erlenmeyer flask (1 L) containing 500 mL of M9+3% NaCl supplemented with 0.5% de maltose (Sigma-Aldrich, St. Louis, USA) or 0.5% maltose+0.5% GlcNAc (Sigma-Aldrich). The flasks were kept in aerobic conditions with gentle shaking at 28°C for up to 72 hours. Aliquots of the bacterial growth culture were taken from the replicates at 24, 44 and 72 hours for the microarray gene expression analysis. The microarray experiments were performed essentially as described previously (16). Bacterial growth was assessed by measuring optical density at 550 nm.

RNA extraction and cDNA preparation

Aliquots (50 mL) of bacterial culture were pelleted and resuspended in 5 mL of TRIzol (Invitrogen, Carlsbad, USA). Nucleic acids were extracted using 2 mL of chloroform (Merck KGaA, Darmstadt, Germany). Samples were incubated for 5 minutes at room temperature and centrifuged at 12000×g (gravity) for 20 minutes at 4°C. Aliquots of 3 mL of the supernatant were placed in clean tubes containing 0.3 mL of sodium acetate (3 M) and 3 mL of isopropyl alcohol (Merck). The tubes were incubated at 4°C for 5 minutes to precipitate the nucleic acids and then centrifuged for 15 minutes at 12000×g and 4°C. In the final step, 2 mL of 70% ethanol (v/v; Merck) was added in order to remove salts. The mixture of nucleic acids was precipitated and treated with DNase I (Invitrogen). Then, 10 µL of DNase I was added to 70 µL of the mixture of nucleic acids (Invitrogen). The mixture was incubated for 1 hour at 37°C. A total of 80 µL acidic phenol (Invitrogen; around pH 5) and 80 µL of chloroform (Merck) were added and the mixture was vortexed for 5 minutes. The mixture was placed in a clean container containing 16 µL of sodium acetate (3 M) and 400 µL of 100% ethanol (v/v) and kept on ice for 5 minutes. It was centrifuged for 15 minutes at 12000×g and 4°C. The supernatant was discarded and 500 µL of 70% ethanol (v/v) was added to wash the RNA pellet. Total RNA was purified using the RNeasy kit (Qiagen, Hilden, Germany). RNA tags were purified using UTPs labeled with an aminoallyl reactive group. This group binds to the dyes Cy3 and Cy5 used to label the samples. cDNA was prepared by mixing 20 µg purified total RNA, 2 µL of random primers (Invitrogen) and 22 µL of RNase-free water. This mixture was incubated for 5 minutes at 70°C and placed on ice. In the next stage 8.0 µL of first-strand buffer (Invitrogen), 2.0 µL of DDT (dithiothreitol; GE Healthcare Biosciences AB, Uppsala, Sweden)

(0.1 M), 4.0 µL of dNTP-aminoallyldUTP and 4.0 µL of reverse transcriptase SuperScriptIII (Invitrogen) were added and the mixture was incubated for 3 hours at 46°C in order to generate a sufficient amount of cDNA. A total of 60 µL of H₂O and 100 µL of phenol-chloroform (Invitrogen) were added and mixed in vigorously. After centrifugation of this mixture of cDNA for 5 minutes at 12000×g, the supernatant was removed and added to a container containing 10 µL of sodium acetate (3 M) and 250 µL of ethanol (100%). The mixture was kept on ice for 5 minutes and centrifuged for 15 minutes at 12000×g and 4°C. The supernatant was removed carefully and 500 µL of ethanol (70%) was added to remove impurities and products not incorporated into the cDNA. The ethanol was removed and the sample was dried at room temperature for 5 minutes.

Cy3 and Cy5 labeling of cDNA and microarray hybridization

The resulting cDNA pellet was labeled with Cy3 or Cy5. To mark the total cDNA with the fluorescent dyes, the pellet was resuspended in 10 µL of sodium bicarbonate (50 mM, pH 9). Ten microliters of Cy3 was added to the sample of cDNA originating from the control (maltose) treatment and 10 µL of Cy5 was added to the sample originating from the test treatment (maltose+GlcNAc). Each sample was incubated for 1 hour in the dark. Excess dye was removed with the aid of SEP columns (Applied Biosystems Life Technologies Carlsbad, USA). The dried labeled cDNA was dissolved in 20 µL of water. The hybridization solution was prepared by mixing 20 µL of cDNA dissolved with 3 µL of sodium dodecyl sulfate 10% (Sigma-Aldrich), 6 µL of yeast tRNA (Invitrogen) and 15 µL of SSC (20×, 3 M NaCl, 0.3 M citrate sodium, pH 7.0). This mixture was incubated for 5 minutes at 96°C, and then immediately incubated for 5 minutes at 60°C. Aliquots of labeled cDNA (10 µL) of each sample (control and test) were mixed and placed on a microarray slide, because the experiments are based on competitive hybridization. The slide contains 4832 ORFs of *V. parahaemolyticus*. The microarray slides were simultaneously prepared to receive the mixture. First, the slides were washed with blocking solution (7.5 g of succinic anhydride, 472.5 mL of 1-Methy-2-pyrrolidinone and 1.0 M sodium borate pH 8.0). Then the slides were washed with water twice for 10 minutes. The slides were washed with absolute ethanol and dried in a hybridization apparatus (MAUI BioMicro Lid Systems, BioMicro, Salt Lake City, USA). The mixtures containing cDNA were added to the slides and kept in the hybridization apparatus for 16 hours at 60°C. The slides were washed with SSC buffer three consecutive times and once with absolute ethanol.

Microarray analysis

After washing and drying, the slides were examined using a scanner, the ScanArray Lite Express (Perkin Elmer Life Sciences, Massachusetts, USA), that enables the detection and quantification of fluorescence. To determine the expression of each gene, log₂ values of fluorescence for the maltose+GlcNAc treatment were divided by those for the control treatment (maltose) for each spot. Gene expression was considered induced or repressed if the fluorescence values were >2 or <-2, respectively, representing a four-fold increase or decrease in fluorescence compared with the control. Dendrograms were constructed using MatLab 7.2.0 on the basis of Euclidian Distance similarity matrices and the Average Linkage clustering method. The localization of the genes in the metabolic pathways was performed using Kyoto Encyclopedia of Genes and Genomes (KEGG).

Results

V. parahaemolyticus RIMD2210633 grown with maltose+GlcNAc showed a biphasic growth curve with two exponential phases (*i.e.* 1–11 hours and 22–27 hours) and two stationary phases (*i.e.* approx. 12–21 hours and 44–72 hours), whereas the control treatment (maltose) resulted in only one exponential phase (*i.e.* 1–11 hours) and one stationary phase (*i.e.* 12–72 hours) (Fig. 1).

Fig. 1. Growth of *V. parahaemolyticus* on maltose and maltose+GlcNAc during 72 hours.

The second exponential phase (22–27 hours) in the maltose+GlcNAc treatment corresponded to the beginning of GlcNAc's utilization by the bacterium. To evaluate the gene expression induced and repressed by GlcNAc, we selected the time points 24, 44, and 72 hours to perform the microarray analysis. Overall, the induced and repressed genes were distributed in three main groups (Figs. 2 and 3). Group one contained genes induced or repressed towards the end of the experiment, between the 44 and 72 hour marks. Group two contained genes induced or repressed mainly in the initial 24 hours of experiment. Group 3 contained genes induced or repressed throughout the 72 hours of the experiment. A total of 81 genes had their expression induced during the experiment; 59 genes at 24 hours, 37 genes at 44 hours, and 31 genes at 72 hours. A total of 39 genes in chromosome I and 20 genes in chromosome II were induced to express by GlcNAc at 24 hours (Table S1). The induced genes encoded mainly proteins related to chemotaxis, substrate binding/transport, GlcNAc metabolism, energy metabolism, unknown processes (hypothetical proteins), and competence/chitin surface colonization (PilA protein). The genes VP0828, VP0829, VP0831, and VPA0038 were associated with the uptake and

metabolism of GlcNAc. These genes were expressed throughout the experiment (Fig. 2). VPA0038 encodes NagB which transform GlcN-6P in Fru-6P (fructose-6-phosphate) and NH₃. Fru-6-P subsequently enters the glycolysis pathway. A NagC repressor (VP0828) and a hypothetical protein (VP0830) inside the *nag* operon were induced too. Overall, genes related to chemotaxis, substrate binding/transport, and GlcNAc metabolism predominated.

A group of 55 genes had their expression repressed during the experiment; 38 genes at 24 hours, 24 genes at 44 hours, and 21 genes at 72 hours (Fig. 3). The genes repressed at 24 hours (26 genes in chromosome I and 12 genes in chromosome II) mainly encoded hypothetical proteins (*e.g.* VP0711, VP2388, VP2400, VP2487, and VPA0055) (Table S2). A putative operon comprising VP0583 and VP0584 was repressed in the presence of GlcNAc. VP0584 (an Isocitrate lyase) is responsible for the transformation of isocitrate in succinate and glyoxylate at a bifurcating point of the Krebs cycle (TCA). This point (glyoxylate pathway) is a bypass of two stages of the TCA, converting isocitrate in succinyl-CoA. Several genes involved in the degradation of starch (VPA0461), maltose (VPA1619), and glycogen (VPA1638 and VPA1641) were also repressed in the presence of GlcNAc throughout the experiment (Fig. 3).

Discussion

Chitin influences the growth and physiology of vibrios in the environment. Chitin may support a significant portion of marine bacterial productivity. The omnipresence of chitin in the marine environment might explain the widespread distribution of vibrios (22, 38). In the present study it was showed that chitin monomers (GlcNAc) also have a significant effect on the growth and physiology of *V. parahaemolyticus* as they induced or repressed genes associated with different cellular processes. For instance, a chitinase (VPA0055) was induced after 24 hours. However, there was no chitin in the medium. We hypothesize that the

Fig. 2. Induced genes during the experiment. The genes induced to express at 24, 44, and 72 hours are represented by three columns. The legend on the left indicates the level of induction. The gene number and product are shown on the right. The cluster was constructed using Euclidian distance similarity matrices and the Average Linkage clustering method. Highly induced genes appear in blue. Numbers on the right correspond to the three main groups of induced genes.

Fig. 3. Repressed genes during the experiment. The genes whose expression was repressed at 24, 44, and 72 hours are represented by three columns. The legend on the left indicates the level of repression. The gene number and product are shown on the right. The cluster was constructed using Euclidian distance similarity matrices and the Average Linkage clustering method. Highly repressed genes appear in brownish red. Numbers on the right correspond the three main groups of repressed genes.

presence of GlcNAc in the bacterial cell might function as a signal to the bacterium, indicating the need to be prepared for a possible chitin-rich environment. VP2487 encodes a N,N-diacetylchitobiose phosphorylase, a novel enzyme described in *V. furnissii* which promotes the reaction of chitobiose (GlcNAc₂) in NAG-1P and GlcNAc (30, 37). In *V. cholerae*, GlcNAc induces the expression of the GlcNAc-binding protein VCA0811, and a chemotaxis protein (VC0449). These facts further support our hypothesis. We suggest that the genes VP2487, VPA0055 and VP2629 belong to the *nag* regulon in *V. parahaemolyticus*. Because the gene organization and the response of the *nag* operon to GlcNAc in *V. parahaemolyticus* and *V. cholerae* is similar, we consider that the overall *nag* regulon was similar and NagC repressor dependent.

Potential operons (*e.g.* the operon composed of VP0710 and VP0711; that composed of VPA1086 and VPA1087; and that composed of VPA002, VPA0004, and VPA0003) were induced during the experiment. VP0710 encodes a membrane protein IIBC specific for trehalose (*treP*) of the PTS system. It transports trehalose into the cell and phosphorylates it. The intracellular trehalose-6-P is processed by trehalose-6-P hydrolase (VP0711, *TreC*) to produce glucose-6-P. In *E. coli* and *B. subtilis*, *treC* and *treP* are organized in an operon induced by trehalose 6-P and negatively regulated by catabolic repression and by the repressor *TreR* (1, 8). The shortage of glucose in the medium might have been a signal for derepression. Another potential operon (VPA1086 and VPA1087) encodes the proteins ribose ABC transport (RbsA) and a ATP-binding protein (RbsD), respectively, which are responsible for the assimilation of R-ribose into the cell. VPA0003 (*OppF*) and VPA0004 (*OppD*) may form an operon responsible for the transport of peptide and nickel into the cell, whereas VPA002 (hypothetic protein), VPA003 and VPA004 may

compose an induced operon of unknown function.

Repressed genes are involved in energy metabolism

V. parahaemolyticus clearly represses the expression of a set of genes in response to GlcNAc. Some of these genes were repressed during the entire experiment. For instance, the Pululanase VPA1638 hydrolyses glycogen by breaking α -1,6-glycosidic bonds. The linear malto-oligosaccharides made available are then hydrolysed in maltose and maltotriose by the maltogenic amylase (VPA1641) (28). The gene VPA1619 encodes a 4- α -glucanotransferase which transforms maltose in α -D-glucose. The repression of these genes indicates a change during growth. After utilizing maltose, the bacterium may have repressed the expression of these genes in order to enter a second exponential growth phase as revealed in previous studies of the utilization of sugar mixtures by *E. coli* (7, 24).

The expression of genes associated with mechanisms of protein folding and secretion was also repressed. The gene VP0510 encodes a DsbC protein which along with the protein DsbA has a key role in the bisulfet bonds of newly formed proteins in the periplasm (46). The gene VP0535 encodes a membrane lipoprotein peptidase involved in the recognition and removal of signal peptides of lipoproteins prior to export. The gene VP1106 encodes LolA, a protein involved in the localization and fixation of the most abundant lipoprotein (Lpp) of the outer membrane (25). The relative repression of protein folding and secretion-related genes may be due to the two different growth phases. With the maltose+GlcNAc treatment, the cells are preparing to enter a new exponential phase, whereas with the control (maltose) treatment they are in a stationary phase where secretion is supposed to be greater.

The presence of GlcNAc may have resulted in the repression of glycolysis-related genes (VP0356 and VPA1230).

VP0356 encodes a pyruvate kinase I which transforms phosphoenol-pyruvate in pyruvate, whereas VPA1230 encodes a glyceraldehyde 3-P dehydrogenase (Gap) which transforms glyceraldehyde-3P in glycerol-1,3P₂. The gap gene is induced in the presence of glucose in *B. subtilis* (42). It would be reasonable to assume that glucose was not available in the present experiment as no Gap induction was observed. Another important effect of GlcNAc on the metabolism of *V. parahaemolyticus* is on the TCA cycle. The repressed malate synthase gene (VP0583) codes for the enzyme that transforms glyoxylate and acetyl-CoA in malate and the isocitrate lyase gene (VP0584) codes for the enzyme responsible for the transformation of isocitrate in succinate, suggesting that the glyoxylate pathway was not active. The glyoxylate pathway allows the production of a molecule with four carbons using two molecules of acetyl-CoA, spending only one CO₂ molecule (23, 29). The activity of the isocitrate lyase directs the TCA cycle towards a more efficient biochemical path for bacterial growth. The glyoxylate pathway is repressed during cultivation in glucose and is induced in cultures with acetate in *E. coli* (12, 28). If we assume that there is a high intracellular content of acetate in the maltose+GlcNAc treatment, *E. coli* and *V. parahaemolyticus* respond differently to similar conditions. This may also suggest that the TCA regulation in *V. parahaemolyticus* is different. Here we assume that there is a shortage of glucose but still a low intracellular level of acetate in the maltose +GlcNAc treated cells at 24 hours and *V. parahaemolyticus* adapts from glucose to GlcNAc utilization.

Two hypothetical operons (VP0081, VP0082, and VP0083; VP0086, VP0087, VP0088, and VP0089) were repressed in the presence of GlcNAc. The response of these genes in the experiments indicates their possible roles in *V. parahaemolyticus* cell physiology.

Induction by GlcNAc may promote horizontal gene transfer (via competence) and the expression of pathogenicity genes in V. parahaemolyticus

A type IV pilin used to produce a type IV pilus was detected in this study. Competence is induced in *V. cholerae* by chitin. The pilus IV complex, DNA-binding proteins, and three regulatory cascades which are affected by the environment and cell physiology, are necessary in order for competence to take place. However, GlcNAc is not able to induce this mechanism in *V. cholerae* (26). A similar process could take place in *V. parahaemolyticus* but in response to GlcNAc. Another induced gene was VP2699, encoding a mannose-sensitive hemagglutinin pilus (MSHA) which is part of the biogenesis of the type IV pilus. MSHA stimulates adherence of *V. cholerae* on various surfaces (e.g. chitinous surfaces, intestinal mucus and abiotic surfaces), and is a key element in biofilm production *V. cholerae* (43). This gene contributes to the formation of a biofilm in *V. parahaemolyticus* (15). Several genes encoding structural and biosynthetic components related to MSH-pilus (24 hours: VP2693 putative MshP protein, VP2694 putative Type IV prepilin, MshO VP2698 putative V10 pilin, VP2699 putative MSHA biogenesis protein MshF, VP2705 MSHA biogenesis protein MshK, VP2706 MSHA biogenesis protein MshJ, and VP2709 single-strand binding protein; 44 hours:

VP2692 hypothetical protein, VP2693 putative MshP protein, VP2694 putative Type IV prepilin MshO, and VP2709 Single-strand binding protein; 72 hours: VP2694 putative Type IV prepilin MshO, VP2698 putative V10 pilin, VP2706 MSHA biogenesis protein MshJ) were expressed slightly below the signal threshold (between -1.1 and -1.9), indicating the induction of several related genes. In addition, a pathogenicity-related gene (VP0704) was also expressed. VP0704 encodes MetQ (YaeC), an antigenic protein of *V. vulnificus*, activated via Toll receptors (21). The induction of competence genes may influence the clonality of vibrios (10, 39)

The present study provides new information on the gene expression of *V. parahaemolyticus* grown on GlcNAc. This aminosugar promotes cell growth and influences the physiology of this *Vibrio* by interfering with the gene expression associated with different cellular processes, most notably the processes for scavenging energy and genome plasticity.

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