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Gene Expression Analysis of *Corynebacterium glutamicum* Subjected to Long-Term Lactic Acid Adaptation▽¶

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Corynebacteria form an important part of the red smear cheese microbial surface consortium. To gain a better understanding of molecular adaptation due to low pH induced by lactose fermentation, the global gene expression profile of *Corynebacterium glutamicum* adapted to pH 5.7 with lactic acid under continuous growth in a chemostat was characterized by DNA microarray analysis. Expression of a total of 116 genes was increased and that of 90 genes was decreased compared to pH 7.5 without lactic acid, representing 7% of the genes in the genome. The up-regulated genes encode mainly transcriptional regulators, proteins responsible for export, import, and metabolism, and several proteins of unknown function. As much as 45% of the up-regulated open reading frames code for hypothetical proteins. These results were validated using real-time reverse transcription-PCR. To characterize the functions of 38 up-regulated genes, 36 single-crossover disruption mutants were generated and analyzed for their lactic acid sensitivities. However, only a *sigB* knockout mutant showed a highly significant negative effect on growth at low pH, suggesting a function in organic-acid adaptation. A *sigE* mutant already displayed growth retardation at neutral pH but grew better at acidic pH than the *sigB* mutant. The lack of acid-sensitive phenotypes in 34 out of 36 disrupted genes suggests either a considerable redundancy in acid adaptation response or coincidental effects. Other up-regulated genes included genes for ion transporters and metabolic pathways, including carbohydrate and respiratory metabolism. The enhanced expression of the *nrd* (ribonucleotide reductase) operon and a DNA ATPase repair protein implies a cellular response to combat acid-induced DNA damage. Surprisingly, multiple iron uptake systems (totaling 15% of the genes induced ≥2-fold) were induced at low pH. This induction was shown to be coincidental and could be attributed to iron-sequestering effects in complex media at low pH.

Most bacteria periodically encounter life-threatening stresses in a variety of environments. To increase survival chances due to sudden changes in, e.g., temperature, pH, or nutrition, bacteria turn on a programmed mechanism that includes the synthesis of stress-inducible proteins (11). Challenges due to an acidic environment are experienced by both pathogenic and fermentative microorganisms, as well as those living "next" to fermentative organisms. Therefore, mechanisms allowing successful adaptation of microorganisms to low pH are essential for survival and proliferation in certain niches. One major biotechnical application creating low-pH niches is cheese making. The curd acidifies through the action of lactic acid bacteria converting lactose to lactic acid. Subsequently, yeasts raise the pH by different metabolic activities, allowing growth of the ripening flora. The ripening flora of many cheeses contains several *Corynebacterium* species (4). For our study, we chose *Corynebacterium glutamicum*, since the complete genome sequence and microarrays are available. *C. glutamicum* (23) is a high-G+C-content aerobic gram-positive bacterium mainly found in soil. It also has significant industrial importance with respect to the production of amino acids, in particular, L-glutamate and L-lysine, which are used as nutritive additives in food and feed (20).

Acid stress includes the combined effects of low pH caused by inorganic acids and/or organic acids present in the environment (1). Uncharged organic acids can passively diffuse across the cell membrane and dissociate in the cytoplasm into negatively charged molecules and protons, thereby lowering the cytoplasmic pH (49). The lower the external pH, the more undissociated organic acid is available to cross the cellular membrane (1). The maintenance of cytoplasmic pH homeostasis under such conditions is crucial for the uninterrupted function of the bacterial cell. Deviations of more than 1 pH unit from the optimal cytoplasmic pH cause considerable changes in cellular functions, damaging enzymes and DNA (12).

An adaptive acid response generated by moderately low pH enables bacterial cells to survive more extreme acidity. This adaptive process, termed the acid tolerance response (ATR), has been extensively studied in a number of gram-positive bacteria, namely, *Listeria monocytogenes* (10), *Lactococcus lactis* (13), *Propionibacterium freundenreichii* (22), and *Streptococcus mutans* (28). While the molecular response to acid shock

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has been studied in depth from a short-term perspective, the long-term adaptation, which is expressed during logarithmic growth at low pH, has been studied to a much lesser extent. Generally, only a limited number of reports that focus beyond a short-term shock response and target the adaptation response are available (31). Interestingly, in the case of UV stress, the UV shock response of the cyanobacterium *Nostoc commune* is fundamentally different from the long-term adaptation response (9). The same was found for cold shock and cold adaptation of *Yersinia enterocolitica* (5). We therefore focused our study on the long-term adaptation response of *C. glutamicum* under acidic growth conditions. Since intracellular regulation of survival, growth, and differentiation should be reflected in altered patterns of gene expression, we aimed at looking into the adaptation response of *C. glutamicum* using physiological and molecular approaches focused on the analysis of stress-induced transcription.

MATERIALS AND METHODS

Bacterial strains, culture media, and growth conditions. *C. glutamicum* strain ATCC 13032 and its derivatives were grown at 30°C in tryptic soy broth (TSB) (BD, Heidelberg, Germany). When appropriate, 50 μg/ml kanamycin (Gibco, Heidelberg, Germany) was added. *Escherichia coli* cells (TOP 10; Invitrogen, Heidelberg, Germany) were routinely grown at 37°C in LB medium. Media used for the acid adaptation experiments were acidified by the addition of 90% (vol/vol) lactic acid (Merck, Ismaning, Germany).

Continuous cultivation. Continuous cultures of *C. glutamicum* were grown in a 2-liter turbidostat fermentor (Biostat B; Sartorius BBI Systems, Melsungen, Germany) at 30°C. The oxygen concentration was kept at 50% of saturation by continuous aeration (2 liters/min) with a mixture of air and nitrogen. The fermentor was inoculated with 2% of an overnight culture. Once the optical density at 600 nm (OD_{600}) reached 0.5, it was kept constant by continuous addition of TSB. During fermentation, the pH was kept constant by adding 1 M NaOH (for pH 7.5) and 1 M lactic acid (for pH 5.7). Samples (50 ml) were drawn after five generations of growth at the desired pH. For this purpose, the generation times were calculated based on the consumption of medium. Cells were harvested by centrifugation (5,000 × *g*; 5 min at 4°C) after the addition of bacterial RNA Protect (QIAGEN, Hilden, Germany) and frozen in liquid nitrogen. The pellets were stored at −70°C until RNA extraction.

Batch cultivation. To determine minimal doubling times and biomasses, *C. glutamicum* was grown in aerated bottles with 100 ml TSB adjusted with lactic acid. The OD_{600} was measured every 15 min. Doubling times were determined from the exponential growth phase. Biomass was determined after vacuum drying of pelleted cells.

Measurement of cytoplasmic pH. Cytoplasmic pH was determined using the fluorescent pH indicator 2′,7′-bis-(2-carboxyethyl)-5[and-6]-carboxyfluorescein (BCECF) as previously described (35) with some modifications. Six milliliters of *C. glutamicum* suspension grown as a batch in TSB at pH 7.5 was harvested at exponential phase (OD_{600} = 0.5) by centrifugation (5,000 × *g*; 22°C; 3 min). The cells were washed two times with 50 mM potassium phosphate buffer (pH 7.5) and resuspended in 3 ml of the same buffer. Thirty microliters of the lipophilic acetoxymethyl ester of BCECF dissolved in dimethyl sulfoxide (0.1 mM) was added to the cell suspension (1 μM end concentration), which was then shaken at 30°C and 200 rpm in the dark for 30 min. The cells were harvested by centrifugation, washed two times with phosphate buffer, and resuspended in phosphate buffer energized by 50 mM glucose at the appropriate pH value. The fluorescence excitation of intracellular BCECF was recorded using a multilabel counter (VICTOR; EG&G Wallac, Turku, Finland). The dual-excitation wavelengths for BCECF were 450 nm (pH-independent isosbestic point) and 490 nm, and the emission wavelength was 535 nm, measured over time intervals of 1 s. The normalization using the fluorescence intensity ratio at the isosbestic point eliminated the fluorescent measurement artifacts, including photobleaching, leakage, and nonuniform loading of the pH indicator. In situ calibration was performed by using a mixture of the ionophores nigericin, gramicidin, and valinomycin (final concentrations, 10, 20, and 10 μM, respectively) to equilibrate the intracellular pH with the controlled extracellular medium between pH 3.5 and pH 7.5. The cytoplasmic pH was estimated using a calibration curve obtained from the normalized emission values correlated with the pH values of the medium.

DNA microarray analyses. The generation of whole-genome DNA microarrays (52), synthesis of fluorescently labeled cDNA from total RNA, microarray hybridization, washing, and data analysis were performed as described previously (21, 26, 39). Briefly, microarray scans were analyzed using the software ImaGene 6.0 (Biodiscovery Inc., CA), and local background median correction, as well as global lowess normalization using all spots, was applied. The signal median was measured in combining spot replicates. Genes that exhibited significantly changed mRNA levels ($P < 0.05$ in a Student's *t* test) by at least a factor of 2 were determined (40).

Validation of expression profiles of fermentation by real-time reverse transcription (RT)-PCR. A 5-ml overnight culture was inoculated (1:200) in 100 ml TSB, pH 7.5 and 5.7. At an OD_{600} of 0.5, 10 ml cell suspension was added to 20 ml of bacterial RNA Protect (QIAGEN, Hilden, Germany) and harvested by centrifugation at 5,000 × *g* for 5 min. The pellets were immediately frozen in liquid nitrogen. Total RNA was isolated using an RNeasy tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations with the following modifications. The frozen cells were resuspended in 700 μl RTL buffer (provided with the RNeasy kit) containing mercaptoethanol. The cells were disrupted three times in a Ribolyzer (Hybaid, Heidelberg, Germany) using Lysing Matrix B beads (Qbiogene, Heidelberg, Germany) for 45 s at a speed of 6.5 m/s. The cells were cooled on ice between runs. Finally, the tubes were centrifuged for 2 min at full speed (16,100 × *g*), and the supernatant was removed and processed according to the manufacturer's recommendations. On-column DNase digestion was performed for 15 min at 30°C, and the RNA was eluted in 50 μl DNase- and RNase-free water. Despite optimization of the DNase treatment, some DNA contamination remained and was removed by a separate DNase digestion using Ambion's DNA-free (Ambion, Cambridgeshire, United Kingdom) according to the manufacturer's instructions.

A two-step real-time RT-PCR was performed. One microgram of total RNA was transcribed to cDNA with Superscript III (Invitrogen, Karlsruhe, Germany) in a 20-μl reaction mixture using random primers at a concentration of 150 ng/μl according to the manufacturer's recommendations. cDNA synthesis was performed for 1.5 h at 50°C, followed by an enzyme inactivation step for 15 min at 70°C. The cDNA was 10-fold serially diluted, and real-time PCR was performed. Changes in gene expression were quantified in an iCycler (Bio-Rad, Munich, Germany). For a 25-μl reaction mixture, 12.5 μl ABsolute QPCR SYBR Green Fluorescein mix (Abgene, Hamburg, Germany), 0.5 μM of each primer amplifying a fragment of 100 to 150 bp, and 2 μl of cDNA were used. The cycling conditions were 40 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 20 s, preceded by a 15-min enzyme activation at 95°C. For each amplification run, the calculated threshold cycle of the 16S rRNA was used for normalization (37). The formation of nonspecific products was excluded by using the melting curve function of the iCycler software version 3 (Bio-Rad, Munich, Germany).

Verification of cotranscribed genes. For verification of cotranscription, 100 ng/μl of total RNA was transcribed to cDNA with Superscript III (Stratagene) in a 20-μl reaction using 2 pmol of each of the primers (see Table S1 in the supplemental material). The reaction was performed for 1 h at 55°C, followed by an enzyme inactivation step for 15 min at 70°C. Subsequently, 2 μl of the cDNA reaction mixture was used for a PCR to verify product formation.

Construction of gene disruption mutants. Primers were designed to amplify an internal ~300-bp fragment of the targeted open reading frame (ORF) (see Fig. S1 in the supplemental material). The fragments obtained were ligated into pCR2.1-TOPO (Invitrogen, Karlsruhe, Germany) and transformed into chemically competent *E. coli* TOP 10cells (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. To select for transformants containing the desired PCR fragments, the cells were plated on LB plates containing 40 μg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 50 μg/ml kanamycin after 1 h of incubation at 37°C. The plasmids containing the PCR fragments were isolated using the Nucleospin plasmid isolation kit (Macherey Nagel, Düren, Germany), and the inserts were verified by PCR. The plasmids were transformed into *C. glutamicum* as previously described (50). Since the plasmid cannot replicate in *C. glutamicum*, it undergoes a homologous crossover, thereby creating a disruption in the reading frame of the ORF (see Fig. S1 in the supplemental material). Gene disruption mutants were selected by plating the cells on LBHIS (tryptone, 5 g/liter; yeast extract, 2.5 g/liter; NaCl, 5 g/liter; brain heart infusion [BHI] powder, 18.5 g/liter; separately autoclaved sorbitol, 91 g/liter) (50) containing 25 μg/ml kanamycin. The integration of the plasmid was confirmed by PCR, using a universal primer located on the plasmid and primers upstream of the homologous recombination site and by Southern blotting with the digoxigenin-labeled TOPO cloning vector on an EcoRI-digested chromosomal DNA.

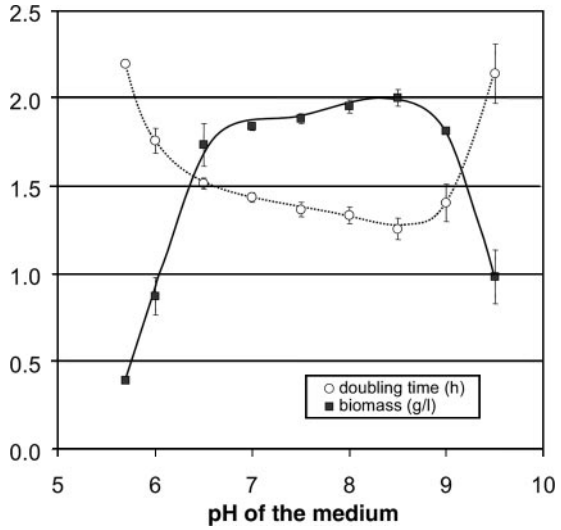

FIG. 1. Comparison of pH values, doubling times, and biomasses of *C. glutamicum* batch cultures in aerated bottles. No growth was observed below pH 5.5 or above pH 9.5. The values of pH 5.7 and 7.5 were chosen for experimental and control conditions, respectively. The error bars represent standard deviations.

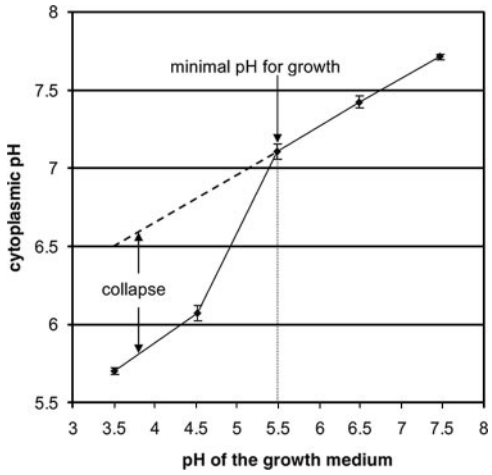

FIG. 2. Cytoplasmic pH as a function of extracellular pH. Cytoplasmic pH changes were measured in exponentially growing *C. glutamicum* ATCC 13032 at pH 7.5 using the fluorescent dye BCECF. The cytoplasmic pH decreased moderately until an outer pH value of 5.5 was reached. Beyond this point, a sudden decrease of the internal pH, indicating a collapse in the cytoplasmic pH homeostasis, was observed. Mean values, with standard deviations, of three experiments are given.

Determination of the growth characteristics of the mutant strains. Mutant strains were analyzed using a honeycomb microwell plate reader (Bioscreen C; Thermo Labsystems, Dreieich, Germany). A 5-ml overnight culture was diluted 1:50 in TSB (pH 7.2), and 10 μl was inoculated in honeycomb plates containing 240 μl of TSB at pH 7.5 or pH 5.7. The plates were incubated (with continuous shaking at 30°C), and OD_{600} measurements were obtained every 15 min for a period of 24 h. The doubling time was determined using the following procedure. An exponential trend line and its R^2 value (coefficient of determination) were calculated. Data points were successively removed from the beginning or the end of each growth curve to reveal the specific time frame in which R^2 reached 99.9% or better.

Acid shock survival assay. Acid shock survival experiments were performed either to determine survival after induction of an ATR in the wild type or to determine the effect of a disrupted gene on survival capabilities under acidic conditions. One milliliter of an overnight culture was inoculated in 100 ml TSB, pH 7.5. At an OD_{600} of 0.5, 50 ml cells was harvested by centrifugation at 5,000 × *g* for 5 min. The cells were suspended in 50 ml TSB, pH 3.5, for 30 and 90 min (in the case of the wild type) or pH 4 for 30 min (in the case of mutants) and incubated at 30°C. One hundred-microliter samples were removed before and after acid shock; serially diluted in TSB, pH 7.5; and plated on tryptic soy agar plates. The survival percentage was calculated by comparing the cell counts obtained following acid shock to those in the original pH 7.5 cell suspension prior to acid shock.

Induction of expression of iron-responsive genes. The prominent induction of iron-responsive genes (four operons) induced at low-pH growth was surprising. An iron assay was performed to assess the availability of iron at low pH in undefined complex growth medium, such as TSB, compared to a defined minimal medium like CGXII. Three different growth media were prepared: TSB, TSB plus Fe^{2+} (containing 0.036 mM of $FeSO_4 \cdot 7H_2O$ and 0.1 mM protocatechuic acid), and CGXII. CGXII was prepared as described previously (25), except that protocatechuic acid was added to a final concentration of 0.1 mM. Protocatechuic acid acts as a chelator, facilitating the bioavailability of iron in the growth medium (29). The pH was adjusted to either 7.5 or 5.7. One hundred milliliters of medium was inoculated 1:100 with an overnight culture. At mid-exponential growth phase (OD_{600} = 0.5), cells from 10 ml medium were harvested by centrifugation at 5,000 × *g* for 5 min. The pellets were immediately frozen in liquid nitrogen and, after RNA isolation, were subjected to real-time PCR analysis as described above.

Iron-sequestering assay. To test for iron sequestering at low pH in complex medium, 5× TSB (BD, Heidelberg, Germany) was prepared. Each batch was divided and adjusted to either pH 5.7 or pH 7.5. The medium was kept at least 1 day at 4°C to allow complete iron sequestering. The medium (eight tubes, 39 ml each) was ultracentrifuged for 24 h at 65,000 rpm in a type 70 Ti rotor (Beckman), which corresponded to a maximal *g* force of 435,000. After centrifugation, the content of each centrifuge tube was divided into four fractions of 9.5 ml each: bottom, lower, upper, and highest. The fractions of the eight tubes from a run were combined accordingly, and the iron content was measured by inductively coupled plasma optical emission spectrometry (Optima 3000; Perkin Elmer). This procedure was carried out twice.

RESULTS

ATR and acid-induced changes of cytoplasmic pH of *C. glutamicum*. To gain an overview of the ability of *C. glutamicum* to grow at different pHs, batch cultures were grown at various pH values ranging from 5 to 10. The minimal pH tolerated was found to be 5.5 (data not shown). For this reason, pH 5.7 was chosen for subsequent acid stress experiments. The shortest doubling times were found around pH 6.5 to pH 8; therefore, pH 7.5 was chosen as a near-neutral control. The upper pH growth limit was recorded at pH 9.5. The doubling times and the biomass productions at different pH values showed an inverse relationship (Fig. 1).

Cytoplasmic pH changes induced by external low pH were determined using the pH indicator dye BCECF (Fig. 2). The cytoplasmic pH decreased only moderately until an external pH of 5.5 was reached. Then, a sudden decrease in cytoplasmic pH was observed, indicating a collapse of the internal pH homeostasis system of the cell. This observation correlates well with the fact that the pH minimum for growth of *C. glutamicum* is at pH 5.5. The ATR of *C. glutamicum* was investigated under different pH conditions. Cells growing exponentially at the control pH of 7.5 and at adaptive pHs of 6.0 and 5.7 were exposed to pH 3.5 for up to 90 min. In the nonadapted culture (pH 7.5), no viable cells were detected after 30 and 90 min of exposure, whereas in the adapted cultures (pH 6.0 and pH 5.7), 10% and 80% survival, respectively, was observed after 30 min of exposure at pH 3.5 (Table 1). Therefore, cells adapted to pH

TABLE 1. Survival of adapted and nonadapted *C. glutamicum* cells upon acid shock to pH 3.5[a]

Time (min) of pH shock	% Survival		
	pH 7.5→pH 3.5	pH 6→pH 3.5	pH 5.7→pH 3.5
0	100 ± 0	100 ± 0	100 ± 0
30	0.001 ± 0	8.4 ± 1.1	70.4 ± 11.7
90	0.001 ± 0	0.015 ± 0.008	0.225 ± 0.054

[a] Surviving cells were determined by serial plating. The data represent means from two independent experiments.

5.7 demonstrate a significant increase in viability when shocked at pH 3.5.

Comparison of gene expression at acidic and neutral pHs. To identify differentially expressed genes in response to acid adaptation, the global gene expression pattern of exponential-phase *C. glutamicum* cells, cultivated in a continuous turbidostat fermentor under neutral and acidic conditions with lactic acid, were analyzed using DNA microarrays (52). Based on the experiments reported above, a growth pH of 5.7 was chosen for the experiments performed under acidic conditions. Starting from two independent cultivation experiments, four DNA microarray analyses were carried out. Genes whose hybridization signal intensities exceeded the background intensity by at least threefold, that were differentially expressed in at least three of the four experiments, and that exhibited median ratios of ≥2 between experiment and control were examined. A total of 116 genes showed higher expression and 90 genes showed lower expression than with growth at pH 7.5, representing about 7% of the ORFs in the genome. These genes are listed in Tables S2 and S3 in the supplemental material. Based on their putative functions, the acid-induced genes can be classified into three main categories: transporters (30 ORFs), transcriptional regulators/proteins (6 ORFs), and cell metabolism proteins (27 ORFs). However, hypothetical proteins of unknown function (i.e., 53 putative genes) comprised as much as 45% of the acid-induced ORFs. Thirty-eight genes with the highest levels of up-regulated expression (compared to Table 2) were chosen for gene disruption analysis (see below).

Thirty ORFs, which are organized in five putative operons, encode putative proteins belonging to different transport systems; four of these operons (NCgl0378 to -0380, -0482 to -0484, -0635 to -0639, and -0773 to -0779) are similar to an ABC-type cobalamin/Fe^{3+} siderophore transport system, and one operon (NCgl0909 and -0910) codes for a predicted ABC-type multidrug transport system. Another group comprises putative proteins involved in the transport of ions: cation transport ATPase (NCgl0375, NCgl1488, and NCgl2463), Co/Zn/Cd efflux (NCgl1232), and a divalent heavy-metal cation transporter (NCgl1379).

Six putative transcriptional regulators were recognized among the up-regulated ORFs: two sigma factors, *sigB* and *sigE*; the sensory component of histidine kinase (NCgl0911); an Mn-dependent transcriptional regulator (NCgl2441); and two putative transcriptional regulators (NCgl0430 and -1019). The remainder of the up-regulated ORFs represent proteins involved in numerous metabolic pathways, such as carbohydrate metabolism and components of respiratory metabolism.

Significantly, subunits of F_0F_1 ATP synthase (NCgl1159 to -1165) and the heat shock proteins GroES and GroEL (NCgl0572 and NCgl0573, respectively), whose roles in temperature stress are well documented, showed twofold down-regulation. Twenty-five genes encoding ribosomal proteins and several transport systems involved in amino acid and cation transport were also down-regulated.

Validation of the expression profile via real-time RT-PCR. Although cDNA arrays are highly sensitive and produce robust data, array data require confirmation (42). Furthermore, the prominent induction of iron-responsive genes (15% of the genes induced ≥2-fold; see Table S2 in the supplemental material) required an explanation. A fermentor has numerous metallic parts, and during an extended fermentation process, trace amounts of metals could have dissolved in the medium due to low-pH changes. These metal ions might in turn have triggered gene expression, which was suspected based on an increased expression of iron and heavy-metal-related genes. To test the potential effect of such a phenomenon, the expression of some genes was analyzed by real-time PCR in cells grown in batch cultures in glass vessels. An overnight culture of *C. glutamicum* was inoculated in fresh medium at pH 7.5 and 5.7. RNA isolated from exponentially growing cells was reverse transcribed and subjected to real-time PCR analysis using an iCycler. The genes analyzed included *sigB* and *sigE* and ORFs from four putative iron transport operons and a cation efflux transporter. The results showed the same trend that was observed in the microarray analysis (Fig. 3). First, these data support the reliability of our microarray-based transcriptional analysis. Second, the hypothesis that transcription might have been triggered by acid-dissolved metal ions rather than the pH decrease appears to be rather unlikely.

Influence of acidic pH on the growth of disruption mutants. To investigate the functions of up-regulated genes under lactic acid growth conditions, single-crossover knockout mutants of genes upregulated at least fourfold and 11 other interesting genes (including *sigB* and *sigE*) were generated, yielding 36 mutants of 38 chosen genes (Table 2). Two of the candidate genes, *nrdE* and *nrdI*, could not be knocked out even under neutral pH growth. To test for pH sensitivity of the mutants, growth experiments were performed at pH 7.5 and 5.7. The wild-type strain used as a control contained plasmid pWLQ2 (30) in order to exclude potential effects of the kanamycin resistance gene. Growth was determined in honeycomb plates, and the pWLQ2-transformed strain grew with a doubling time of 3 h at pH 7.5 and 8 h 17 min at pH 5.7. Only two mutants, *sigB* and *sigE*, showed a decreased growth rate compared to the wild-type strain when grown at pH 5.7. The *sigB* mutant was more susceptible to pH 5.7 (doubling time, 17 h 39 min) than the *sigE* mutant (doubling time, 12 h 19 min), whereas at pH 7.5, the *sigE* mutant's doubling time (6 h 18 min) was about 1.6-fold longer than that of the *sigB* mutant (3 h 50 min) (Fig. 4).

To further analyze the responses of the mutants to acidic pH, acid shock experiments were performed. Exponentially growing cells were harvested, resuspended in TSB at pH 4, and shocked for 30 min. Samples were taken before and after acid shock, and survival was determined. The *sigE* mutant exhibited an approximately 10-fold and the *sigB* mutant an approximately 1,000-fold reduction in survival compared to the wild-type strain. No significant differences were observed in the acid

TABLE 2. 38 ORFs of *C. glutamicum* used for insertional mutagenesis

NCBI no.	Gene[a]	ORF[b]	Annotation	Ratio[c]
		517[d]	Hypothetical protein	10
NCgl0123		518	Hypothetical protein	7
NCgl0375	*zntA*	851	Cation transport ATPase	2
NCgl0377		854	Hypothetical membrane protein	7
NCgl0380		857	ABC-type transporter; ATPase component, similar to ABC-type cobalamin/Fe^{3+} siderophore transport systems, ATPase components	4
NCgl0381		3543	Hypothetical membrane protein	18
NCgl0430		927	Bacterial regulatory protein; predicted ArsR family transcriptional regulator	5
NCgl0482	*fepC*	3549	ABC-type transporter; ATPase component, similar to ABC-type cobalamin/Fe^{3+} siderophore transport system	5
NCgl0483	*fepG*	3550	ABC-type transporter, permease component, FecCD transport family; similar to ABC-type cobalamin/Fe^{3+} siderophore transport system	4
NCgl0484	*fecCD*	3551	ABC-type transporter, permease component, FecCD transport family; similar to ABC-type cobalamin/Fe^{3+} siderophore transport system	4
NCgl0623		1152	Hypothetical protein	6
NCgl0636	*fepC*	1169	ABC-type transporter, ATPase component; similar to ABC-type cobalamin/Fe^{3+} siderophore transport systems	5
NCgl0637	*fepG*	1170	ABC-type transporter, permease component; similar to ABC-type cobalamin/Fe^{3+} siderophore transport systems	6
NCgl0639	*fhuD*	1173	ABC-type transporter, periplasmic component; similar to ABC-type cobalamin/Fe^{3+} siderophore transport systems	7
NCgl0734		1293	Hypothetical protein, similar to transcription factor WhiB	3
NCgl0773	*viuB*	1346	Siderophore-interacting protein	7
NCgl0774	*fhuD*	1347	ABC-type cobalamin/Fe^{3+} siderophore transport system, periplasmic component	11
NCgl0911		1518	2CS sensory transduction histidine kinase	3
NCgl0943		1558	AraC-type DNA-binding domain-containing protein	8
NCgl1012	*prkA*	1632	Mg-chelatase subunit ChlI	4
NCgl1019	*marR*	1642	Transcriptional regulator	2
NCgl1075	*rpoE*	1703	DNA-directed RNA polymerase specialized sigma subunits, similar to sigma-70 factor (ECF subfamily) and $sigma^{24}$	2
NCgl1232	*czcD*	2779	Co/Zn/Cd efflux system component	7
NCgl1289		2703	Hypothetical protein	5
NCgl1359	*recN*	2607	ATPase involved in DNA repair	2
NCgl1488	*mgtA*	2433	Cation transport ATPase	3
NCgl1646		3347	Hypothetical protein	4
NCgl1844	*rpoD*	2000	DNA-directed RNA polymerase sigma subunit SigB, similar to $sigma^{70}$/$sigma^{32}$ factors	2
NCgl1959	*fhuD*	2146	ABC-type transport systems, periplasmic component; similar to ABC-type cobalamin/Fe^{3+} siderophore transport systems	8
NCgl2441	*troR*	3470	Mn-dependent transcriptional regulator	2
NCgl2443[e]	*nrdE*	3467	NrdE, ribonucleotide reductase alpha subunit	5
NCgl2444[e]	*nrdI*	2930	NrdI, ribonucleotide reduction protein	4
NCgl2450		2920	Hypothetical protein, involved in propionate catabolism	35
NCgl2451		2919	Hypothetical protein	14
NCgl2584		21	Hypothetical protein, involved in biosynthesis of extracellular polysaccharides	5
NCgl2946		1941	Hypothetical protein	4
NCgl2965		3452	Hypothetical membrane protein, similar to permeases of the major facilitator superfamily	5
NCgl2970	*fhuD*	3458	ABC-type transport systems, periplasmic component; similar to ABC-type cobalamin/Fe^{3+} siderophore transport systems	3

[a] Gene names are given according to the closest homolog in the Entrez database.
[b] The ORF number corresponds to the oligonucleotide number used for the microarrays.
[c] The ratios shown are the average ratios of four microarray experiments. The presented integer digits are significant.
[d] This ORF of the original microarray ORF set is not annotated in the database, and therefore no NCgl number has been assigned. The oligonucleotide used in the microarray covers NCgl0122 and NCgl0123, both of which encode hypothetical proteins.
[e] These genes could not be knocked out and are probably essential (see Discussion).

shock survival of any of the other disruption mutants (data not shown).

Analysis of a multidrug efflux transporter operon in conjunction with the flanking 2CS operon. The multidrug efflux operon (NCgl0909-NCgl0910) is located upstream of a two-component system (2CS) (NCgl0911-NCgl0912), and it is separated by a 146-bp intergenic DNA sequence from a putative multicopper oxidase located upstream. Cotranscription analysis with *C. glutamicum* grown in batch culture at low pH showed that the 2CS and the multidrug transporter genes are transcribed as a single transcript (data not shown). The 2CS might play a role in regulating the expression of the multidrug transporter operon under acidic conditions. We investigated the effect of NCgl0911 (the sensory component of the 2CS) on the expression of NCgl0909 using an NCgl0911 disruption mutant. In this mutant, a twofold up-regulation was found compared to the wild-type strain. A disruption mutant of the response regulator gene NCgl0912 led to a significant decrease in expression of NCgl0909 and NCgl0911 (8- and 10-fold, respectively) observed under low-pH conditions, suggesting an en-

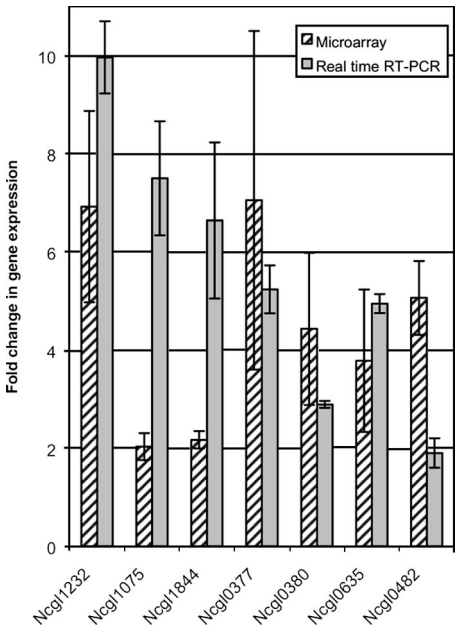

FIG. 3. Comparison between microarray and real-time PCR gene expression analyses in *C. glutamicum*. Gene expression changes in cells adapted to pH 5.7 in a fermentor analyzed by microarray were compared to real-time RT-PCR of *C. glutamicum* cells grown in glass vessels in batch culture. The error bars in real-time-PCR data represent mean deviations obtained from two independent experiments performed in duplicate. The error bars in microarray data represent standard deviations for four microarrays. NCgl1232 (Co/Zn/Cd efflux system), NCgl1075 (*sigE*), NCgl1844 (*sigB*), NCgl0377 (hypothetical membrane protein), NCgl0380 (similar to ABC-type cobalamin/Fe^{3+} siderophore transport systems, ATPase components), NCgl0635 (similar to siderophore-interacting proteins), and NCgl0482 (similar to ABC-type cobalamin/Fe^{3+}-siderophore transport system) are shown.

hancement of transcription by the regulator (RT-PCR data not shown).

Up-regulation of iron transport-related genes. Due to the prominent expression of the iron transport operons (18 genes, comprising approximately 15% of the genes induced twofold and higher; compare Table S2 in the supplemental material), the availability of iron in complex and minimal media was examined. One of the genes present in the iron siderophore operons, NCgl0378, was investigated by real-time RT-PCR for changes in expression levels in cells grown at neutral and acidic pHs in three different media, the commonly used TSB, TSB supplemented with additional iron (TSB plus Fe^{2+}), and one minimal medium (CGXII). In accordance with the microarray data, the level of NCgl0378 expression in TSB was approximately 6.6-fold higher at pH 5.7 than at pH 7.5. Not surprisingly, the expression level in TSB plus Fe^{2+} showed reduced expression (7.4% ± 0.2%) compared to TSB alone. However, after acidification of TSB plus Fe^{2+}, expression of NCgl0378 was again increased approximately sixfold. Cells grown in CGXII minimal medium at near-neutral pH exhibited a similar expression level of NCgl0378 compared to the complex medium TSB plus Fe^{2+} (7.5 ± 0.3%). However, unlike with the complex media, we observed even less expression of NCgl0378 (0.7-fold) after acidification of the minimal medium (Table 3). To exclude the possibility that only TSB triggered the observed behavior, we examined another complex medium (BHI) and found similar results (data not shown). Interestingly, after ultracentrifugation of TSB medium, iron was found in larger amounts in the lower fractions when the medium was acidified (pH 5.7) in comparison to the medium with a nearly neutral pH (pH 7.5) (data not shown).

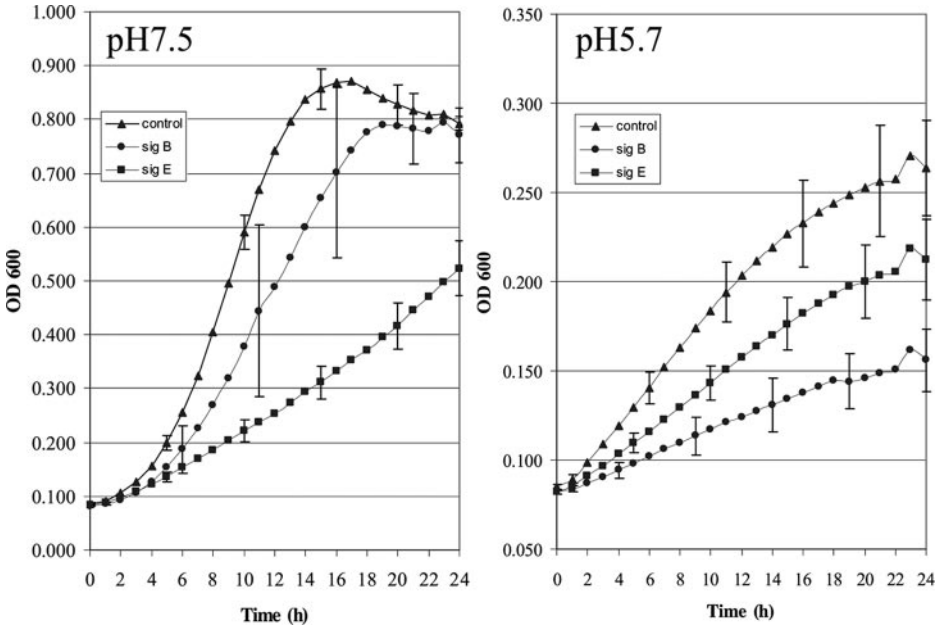

FIG. 4. Growth curves of *C. glutamicum* ATCC 13032 in BHI, showing the wild-type control and the *sigB* and *sigE* mutants. Cells were precultured overnight in TSB, pH 7.5; diluted 50-fold; and inoculated in TSB at pH 7.5 and at pH 5.7. Growth of the cells was monitored in a honeycomb microwell plate reader, Bioscreen C. The error bars represent standard deviations obtained from four independent batches.

TABLE 3. Relative expression of NCgl0378, a gene of the iron siderophore operon from *C. glutamicum*, as determined by real-time PCR[a]

Medium	pH 7.5	Change (*n*-fold) from pH 7.5 to pH 5.7	pH 5.7
TSB	100	6.6→	656.5 ± 104.6
TSB + Fe^{2+}	7.4 ± 0.2	5.7→	42.3 ± 6.6
CGXII	7.5 ± 0.3	0.7→	5.5 ± 0.4

[a] Expression of NCgl0378 in TSB at pH 7.5 was arbitrarily set to 100%; all other data are given relative to this expression level. Mean deviations are given from two independent experiments, each performed in duplicate.

DISCUSSION

ATR. Many, if not all, bacteria mount an ATR upon exposure to mild acidic conditions, enabling the cells to survive low pH values. The most important ATR systems in many bacteria consist of an RpoS/SigB-mediated general stress response and amino acid decarboxylases, which convert their substrates by consuming protons. The product (γ-aminobutyric acid in the case of glutamate) is exported (1, 44). Some bacteria, especially those without a respiratory chain, may use the F_0F_1 ATPase to expel protons at the expense of ATP (see below). Much less, however, is known about long-term acid adaptation. Transcriptional analysis of the ATR and acid adaptation of *Streptococcus pneumoniae* revealed that during the ATR, 38 genes were up-regulated, whereas only 3 specific genes were found for the acid-adapted phase (33). Apparently, once the bacteria reach the adaptation phase through the ATR, only a few genes are required for survival. This is nicely reflected by the fact that, despite knocking out several highly acid-induced genes in *C. glutamicum*, no phenotype concerning acid adaptation was found. This raises the question of whether the changes recorded are protective or just coincidental. At least the iron uptake systems are not protective per se. If the up-regulated genes are indeed protective, one has to propose redundancy for these gene functions (6, 14). Otherwise, up-regulated genes might reflect a function in a slow-growth mode of the cell rather than in acid protection. Due to the different growth rates at acidic and neutral pHs, there is no possibility to differentiate between growth rate control and acid response. However, according to Pieterse et al. (38), at least some of the induced genes should be attributable to acid adaptation.

Induction of transport systems. We have shown that *C. glutamicum* can maintain its cytoplasmic pH over a relatively broad range of external pHs. Bacteria utilize various mechanisms to maintain pH homeostasis within the cell. One of the well-described mechanisms is based on F_0F_1 ATPase, which either produces ATP using the transmembrane proton gradient through proton influx (acidification of the cytoplasm) or expels protons from the cell using the energy provided by ATP hydrolysis (7). In aerobic organisms, such as *E. coli* and *Bacillus subtilis*, the F_0F_1 ATPase mainly functions in ATP synthesis (33). Interestingly, in *L. monocytogenes* the F_0F_1 ATPase is down-regulated during an ATR to save ATP, since these bacteria lack a functional respiratory chain (2). In *C. glutamicum*, the ATPase might be down-regulated to restrict proton influx under acidic conditions. In turn, less ATP can be produced, which may limit growth (compare Fig. 1). However, to pin down the exact meaning of the ATPase down-regulation in *C. glutamicum*, further investigations are needed.

Among the transport proteins induced through acid adaptation were several cation transport ATPase genes (NCgl0375 and NCgl1488). While it is unknown which cations are transported by the encoded proteins, cation transport ATPases, such as K^+ or Na^+ ATPases, have been described in various gram-positive organisms (8, 24) as one of the systems responsible for pH homeostasis, working through a cation-proton antiport mechanism, which leads to alkalinization of the cytoplasm (3).

Increased expression of a putative multidrug transport system (NCgl0909 and NCgl0910) was also observed, which is, at least in part, regulated by the adjacent 2CS (NCgl0911 and NCgl0912). It has been suggested that multidrug efflux systems are part of the natural defense mechanisms of bacteria against toxic compounds, e.g., lipophilic inhibitors, existing in the natural environment (36).

DNA repair. It has been documented that acid stress causes damage to DNA (1). In the microarray experiment of this study, elevated expression of an ATPase involved in DNA repair was observed (NCgl1359). At the same time, we also noticed the up-regulation of the ribonucleotide reductase genes *nrdE*, *nrdI*, and *nrdH* (NCgl2443, NCgl244, and NCgl2445, respectively). In *Corynebacterium ammoniagenes*, the *nrd* genes are arranged in an operon following the pattern of *nrdH-nrdI-nrdE*, and they function in dNTP synthesis in DNA replication and repair, but their specific roles are unclear (48). Attempts to create disruption mutants for *nrdI* and *nrdE* were not successful (data not shown). While this could have been due to technical reasons or the secondary-structure formation of the DNA, previously published data about *C. ammoniagenes* (48) and *B. subtilis* (46) suggest an essential role in cellular function. These two genes are tightly regulated by both cell cycle and environmental cues to provide a sufficient pool of balanced dNTPs for DNA replication and repair (48). In the acid adaptation experiment, exponentially growing control cells at neutral pH grew faster than adapted cells under acidic conditions, but there was a clear difference in the expression levels of *nrd* observed in acid-adapted and nonadapted cells (data not shown). Therefore, the up-regulation of the *nrd* operon, in addition to the DNA ATPase repair protein, suggests a cellular response to combat DNA damage. Similar DNA repair systems induced at low pH have been found in *S. mutans* (15, 17).

Iron transport is not induced by low pH as a primary cause. Surprisingly, numerous iron transport genes were induced at acidic pH. A similar result has been reported for *S. pneumoniae* shocked to pH 6 in complex medium, in which several iron transport genes were also identified among the genes affected by acidification (34). Under aerobic conditions and at neutral pH, iron is present in its insoluble form of Fe^{3+}, whereas low pH increases the solubility of iron by reducing it to Fe^{2+}, thereby increasing the bioavailability of iron in the medium (18). Low pH, therefore, should repress the expression of iron transport systems. Our results, however, suggest a decrease of iron availability in complex media at acidic pH (Table 3). Therefore, it is reasonable to assume that the up-regulation of the iron Fe^{3+} ABC siderophore transporters observed is the result of decreasing iron availability at low pH in the complex medium. Iron can be sedimented in acidified

medium by ultracentrifugation. Since this coincides with the induction of the iron uptake systems in *C. glutamicum*, we propose that iron bioavailability is decreased by an unknown sequestering mechanism at low pH.

Roles of regulatory proteins in acid adaptation. Regulatory proteins play important roles in stress response by directing protein expression in response to environmental fluctuations. We observed the induction of six regulatory protein genes (NCgl0430, NCgl0911, NCgl1019, NCgl1075 [*sigE*], NCgl1844 [*sigB*], and NCgl2441) during acid adaptation.

Transcription of *sigB* (NCgl1844) is strongly induced. The role of its product as a general stress protein has been extensively studied in gram-positive organisms under different stress conditions. Null mutations in *B. subtilis sigB* lead to an increased sensitivity to low pH, heat, and oxidative stress (19). In *L. monocytogenes*, the function of *sigB* has been demonstrated in response to several stresses, such as oxidative stress, osmotic stress, carbon starvation, and growth at low temperatures. For instance, a *sigB* null mutant exhibited a 1,000- to 5,000-fold decrease in survival when exposed to pH 2.5 (53). Studies of *C. glutamicum* CCM 251 showed an effect of *sigB* on the growth and viability of cells under acid, salt, alcohol, heat, and cold stresses (16). Since sigma factors regulate large genetic networks, it is in turn expected that their removal negatively influences growth and survival under any stress. Furthermore, it is expected that the *sigB* gene is more highly expressed with slower growth (27).

NCgl1075 encodes a protein showing homology to SigE, which belongs to the extracytoplasmic function family. SigE's role as an alternative sigma factor regulator was demonstrated, e.g., in *Mycobacterium tuberculosis*, *B. subtilis*, and *Pseudomonas aeruginosa* under heat shock and oxidative stress (41). In *M. tuberculosis*, a *sigE* disruption mutant exhibited higher sensitivity to various environmental stresses compared to the wild type, but not to acidic pH (32, 43). Our phenotypic studies using the *sigE* disruption mutant also did not support a major role in acid stress. Due to the pleiotropic effects of this regulator, growth retardation is already visible at neutral pH and is pronounced at low pH, but less so than in the *sigB* mutant. We speculate that *sigE* is up-regulated by stress factors originating as a consequence of pH stress.

Another regulatory gene that was up-regulated is NCgl0911, encoding the sensory component of a two-component histidine kinase system. 2CSs are widespread in bacteria and are known to assist bacterial cells in adaptation to various environmental alterations (47).

Potential redundancy of the pH adaptation of *C. glutamicum*. From the 116 up-regulated genes after adaptation to lactic acid, 38 were chosen for further mutational analysis, including all of those induced fourfold and higher and 11 other interesting genes. However, aside from the two sigma factor gene disruption mutants (*sigB* and *sigE*), no phenotypical effects under low-pH conditions were observed in the remaining 34 mutants. Either this suggests a considerable redundancy in the adaptation response to lactic acid, or these results indicate indirect effects of pH stress, like a diminished growth rate. However, similar data have been reported for other bacteria in studying the effect of single-insertion mutagenesis on acid stress. In analyzing the ATR in *Salmonella enterica* serovar Typhimurium, inactivation of single genes known to contribute to acid stress exhibited only a marginal effect on acid tolerance (45). Other studies involving *S. enterica* serovar Typhimurium showed that inactivation of two or more genes were needed to eliminate acid resistance (14). Similar observations were also reported for *Bacillus cereus*, in which only one acid-sensitive single-knockout mutant was obtained despite screening of 1.7×10^8 cells. Unfortunately, the identity of the mutated gene is not known (6). These and our own observations lead us to suggest that the genes that are responsible for long-term acid adaptation may have overlapping functions. For instance, several ABC transporters are induced under lactic acid conditions, but their roles remain unclear. Therefore, to obtain acid-sensitive mutants, either the disruption of pleiotropic regulatory proteins is needed (as shown with *sigB*) or more than one gene must be deleted from the genome. Furthermore, for some genes (e.g., iron-responsive genes), coincidental expression could be demonstrated, which might also be true for some other genes. In an interesting study, Pieterse et al. (38) recently tried to exclude coincidental gene expression patterns in the transcriptional response of *Lactobacillus plantarum* to lactic acid by using, besides multiple other conditions, lactate at neutral pH in comparison to lactic acid at acidic pH. When they compared lactic acid stress versus two different growth rates (both in 300 mM NaCl), a prominent cluster appeared to be more highly expressed under both conditions. Since we cannot discriminate different growth rates at the different pH values, we might have unintentionally knocked out extra genes responsive to the reduced growth rate; however, we still should have picked genes up-regulated due to lactic acid alone. Unfortunately, no mutants have been generated by Pieterse et al. (38), making it impossible to connect regulation of transcription with any phenotypic effect.

Final remarks. The adaptation response of *C. glutamicum* to lactic acid is of pleiotropic nature, involving the increased expression of 116 genes with various functions. Clearly, not all of the up-regulated genes are dedicated to pH adaptation only. However, there is evidence that many of these genes do help to adapt the organism to the organic acid stress. Subsequent to the ATR, remodeling of the cell composition occurs (51). Once adaptation is achieved, only a minority of genes may be necessary to further cope with low pH, despite having a lower growth rate, probably due to higher energy costs of internal pH maintenance. In general, our work also provides a cautionary tale about the interpretation of changes in the transcriptional patterns recorded by microarrays and various other technologies without connecting these data to further experimental evidence, such as mutational analysis and physiological data.

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