Transcriptional Regulation of the Cytochrome b\textsubscript{562-\text{o}} Complex in Escherichia coli

GENE EXPRESSION AND MOLECULAR CHARACTERIZATION OF THE PROMOTER*

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Transcriptional regulation of the cyo operon coding for a terminal oxidase, the cytochrome b\textsubscript{562-\text{o}} complex, of the aerobic respiratory chain of Escherichia coli was studied using a chromosomal operon fusion technique with the lacZ gene. Expression of the cyo gene was found to be subject to catabolite repression and also to control by the oxygen concentration. Information on the mechanisms of these regulations was obtained by nucleotide sequencing of the regulatory region, which corresponds to the 5\textsuperscript{'}-flanking region of the cyoA gene. A typical promoter sequence and two noteworthy composite structural features, that is, two potential catabolite gene activator protein-binding sites and a region of hyphenated dyad symmetry were found. The transcription start point was identified by primer extension analysis, confirming the promoter site in the nucleotide sequence. The homology search of the nucleotide sequence in the region of hyphenated dyad symmetry showed a conserved nucleotide sequence in six oxygen-regulated genes examined, which suggests that this is the consensus sequence for the regulation by oxygen.

The aerobic respiratory chain of Escherichia coli branches at a ubiquinol site into two terminal oxidases, cytochrome b\textsubscript{562-\text{o}} and cytochrome b\textsubscript{563-\text{o}} complexes, which both catalyze four-electron transfer to molecular oxygen and couple this redox reaction with the generation of a proton motive force across the membrane (1). Mutants lacking either of the oxidases can grow normally, but a mutant lacking both enzymes cannot grow aerobically on nonfermentable substrates such as succinate (2).

These two terminal oxidases have been studied extensively both biochemically and at a molecular level (3). The cytochrome b\textsubscript{562-\text{o}} complex has been purified and shown to consist of two or four polypeptides (4, 5). The two major subunits (subunit I and II) seem to be functionally essential for ubiquinol oxidation and generation of a membrane potential (4). The genes encoding these subunits are located at 10.2 min on the E. coli genetic map (2). The cyo locus was recently isolated (6), sequenced, and shown to contain five open reading frames, cyoABCDE (7). The cyoA gene encodes subunit I (M\textsubscript{r} = 35,000) and the cyoB gene encodes subunit I (M\textsubscript{r} = 65,000) which contains two protohemes, b\textsubscript{562} and o (4, 8).

Despite recent advances in biochemical and molecular biological studies on bacterial aerobic oxidases and related enzymes (1), there have been few investigations on the metabolic control regulating the aerobic/anoxic switch. Iuchi et al. (9, 10) have explored the overall regulation of a number of enzymes of aerobic metabolism and proposed that the genes arcA and arcB, global regulatory genes in E. coli, mediate pleiotropic repression of enzymes in aerobic pathways. Genes that are not expressed under anaerobic conditions but are expressed aerobically have not yet been well characterized. The cyo gene is one such gene as evidenced by spectroscopic (11) and immunological (12) findings that synthesis of cytochrome o is derepressed by high oxygen tension. Broman et al. (13) showed that a cya mutant contains a reduced level of cytochrome o and that the level is restored to normal when the mutant is grown in the presence of cAMP. These results suggest that transcription of the cyo gene is derepressed aerobically and is subject to catabolite repression. Therefore, it is interesting to study the transcriptional regulation of the cyo gene by the operon fusion technique (14) and to compare the nucleotide sequence of its regulatory region with those of other genes controlled by oxygen.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, T4 DNA ligase, DNA polymerase I (Klenow fragment), Exonuclease III, mung bean nuclease, and RAV-2' reverse transcriptase were purchased from Takara Shuzo Co., Kyoto. Modified T7 DNA polymerase and sequencing reagents were from United States Biochemical Corp. Casamino acids and MacConkey medium were obtained from Difco. PIPES was from Dojin Laboratories, Kumamoto, Japan. RNase A, CAMP, ONPG, and other chemical reagents were purchased from Sigma. [α\textsuperscript{32}P]dCTP (111 TBq/mmol) and [γ\textsuperscript{32}P]ATP (148 TBq/mmol) were from ICN Radiochemicals.

Bacterial Strains and Phages—The bacterial strains and phages used in this work are listed in Table I. All strains used for study of cyo gene expression were isogenic to strain 3000X.

Media—For assay of β-galactosidase activity, cells were grown in DM minimal medium (10) supplemented with 1 μg/ml thiamine hydrochloride. Where indicated, sodium lactate, glucose, mannitol, galactose, xylose, or glycerol was added at 0.4% (w/v). Casamino acids were added at 2% (w/v) as a carbon source where indicated. For anaerobic growth, potassium nitrate was present at 40 mM as a terminal electron acceptor. MacConkey maltose-arabinose plates containing 1% (w/v) each sugar was used to screen cyo mutants (18). Antibiotics were used as described by Maniatis et al. (17).

Growth Conditions—All cultures were grown at 37 °C. Cell density was measured with a Coleman colorimeter (model 20A) at 650 nm. When cultures reached the desired density, chloramphenicol was added to 30 μg/ml to stop protein synthesis after cell harvest. Aerobic growth was measured in U.S.A.

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†1 The abbreviations used are: RAV-2, Rous-associated virus 2; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); ONPG, 4-nitrophenyl-β-D-galactopyranoside; kb, kilobase pair(s); CAP, catabolite gene activator protein; bp, base pair(s).
cultures were incubated in a Monod tube with shaking at 24 rpm. Strictly anaerobic cultures were incubated in test tubes filled to the top with medium, sealed with a rubber stopper, and gently stirred with a small magnetic bar. Semianaerobic cultures were incubated in 100-ml flasks by bubbling with nitrogen gas. The oxygen concentrations of samples of cultures were measured polarographically with a Clark-type oxygen electrode (Yellow Springs Instrument Co.). A positive gas pressure allowed sampling with a Gastight syringe (Hamilton Co., Reno, NV) without any contamination by air. As addition of potassium cyanide at 40 mM blocked the oxidase activity (18), consumption of oxygen during manipulations could be ignored. The initial oxygen concentration was assumed to be 250 μM at 37°C.

Genetic Procedures and DNA Manipulations—Transcriptional crosses using phage P1vir were performed as described by Miller (19). Bacterial transformation was carried out by a standard method (20). Preparation of plasmid DNAs, phenol treatment, and other genetic procedures were performed as described by Maniatis et al. (17). Oligonucleotide primers for sequence analysis and primer extension analysis were synthesized in a model 381A DNA synthesizer (Applied Biosystems Inc.).

Construction of Operon Fusion Plasmid—pHN11 is a pUC8 derivative plasmid harboring a BamHI-HindIII (H1) chromosomal fragment of 4.0 kb in the multi-cloning site (8). This chromosomal fragment contains the whole cyoA gene and a 3’-truncated cyoB gene. The strategy used is shown in Fig. 1. The 3.4-kb EcoRI-HindIII fragment from phN11 was inserted into the EcoRI-HindIII site of pBR322. The resulting plasmid pTO33 was digested with HindIII and protein concentration of cell suspensions was determined by the method of Lowry et al. (22) with bovine serum albumin as a standard. The resulting restriction sites and DNA are shown. Plasmid pTO1 was integrated into the genome of E. coli strain ST7538 (polA1, AroC, thi) by homologous recombination, Linkage of amp' and cyo was confirmed by phase P1 transduction. The abbreviations used are: B, BamHI; E, EcoRI; H, HindIII; S, Sal I; f1g, T4 DNA ligase.

DNA Sequencing—A 1.9-kb SalI (SalI/SalII) fragment was excised from pTO1 and recloned into the SalI site of plasmid pUC118 (23). The recombinant plasmid, designated as pPM201, was used for plasmid-directed sequencing of the anti-sense strand and as a starting material for the preparation of deleted derivatives. A deletion series were made by cleavage of the BamHI and SacI sites in the multi-cloning site of pPM201 by digestion with Exonuclease III, and the treatment with mung bean nuclease and Klenow fragment (24). These fragments were then circularized by blunt-end ligation. The plasmids obtained were used as single-stranded template DNA, for sequencing the sense strand. Single-stranded template DNAs were prepared as described by Zhang et al. (25). Sequencing was done by the dideoxy method (26) using [α-32P]dCTP and modified T7 polymerase (27).

Preparation of E. coli Total RNA—E. coli total RNA was isolated essentially by the method of Alba et al. (28). Cells were harvested in the early exponential phase and suspended in 1.5 ml of a solution of 20 mM sodium acetate (pH 5.2), 0.1% (w/v) sodium dodecyl sulfate, and 1 mM EDTA. RNA was recovered by two rounds of phenol extraction at 60°C for 5 min.

Primer Extension Analysis—Primer extension analysis was performed as described by Karrer et al. (29) with the following modifications. Fifty pmol of synthesized primer was mixed with 50 pmol of [γ-32P]ATP and 27 units of T4 polynucleotide kinase in 50 mM Tris (pH 8.0), 10 mM MgCl2, and 10 mM β-mercaptoethanol in a total volume of 100 μl. After incubation at 37°C for 20 min, DNA was precipitated with ethanol and dissolved in 50 μl of hybridization buffer (60% (v/v) formamide, 0.5 M NaCl, 1 mM EDTA, and 40 mM PIPES (pH 6.8)). The mixture was denatured by heating at 60°C for 5 min, then hybridized with 5 pmol of the primer in a reaction volume of 10 μl at 60°C for 30 min. Each primer was added to the reaction mixture after hybridization at 40°C for 30 min to a final concentration of 0.3 pmol. Primer extension reactions were performed with 10,000 cpm of 32P-labeled DNA and 100 pmol of reverse transcriptase. Incubation was at 37°C for 20 min. The products were separated by electrophoresis on a 10% denaturing polyacrylamide gel and autoradiographed. The migration rate of the primer was determined by running the primer alone under the same conditions as the samples. The migration rate of the primer alone was noted as zero on the autoradiogram. Each primer was added to the reaction mixture after hybridization at 40°C for 30 min to a final concentration of 0.3 pmol. Primer extension reactions were performed with 10,000 cpm of 32P-labeled DNA and 100 pmol of reverse transcriptase. Incubation was at 37°C for 20 min. The products were separated by electrophoresis on a 10% denaturing polyacrylamide gel and autoradiographed. The migration rate of the primer was determined by running the primer alone under the same conditions as the samples. The migration rate of the primer alone was noted as zero on the autoradiogram.

### Table I

| Strain       | Relevant genotype | Source         |
|--------------|-------------------|----------------|
| 3000X        | thi, ΔlocX71      | Laboratory stock |
| CA9445       | thi, ΔcyoA, ΔcyoB | Laboratory stock |
| ST501        | polA1, zip-219:Thi10 | Laboratory stock |
| ST4232       | W2002 ΔcyoZ, ΔcyoA | Laboratory stock |
| ST7358       | 3000X polA1       | This study      |
| ST7359       | ST2538 ΔcyoZ, ΔcyoA, ΔcyoB | This study |
| ST7367       | ST2538 ΔcyoZ, ΔcyoA, ΔcyoB | This study |
| ST7371       | ST2538 ΔcyoZ, ΔcyoA, ΔcyoB | This study |

Phage

P1vir Used in transduction experiments

M13K07 Used for preparation of single-stranded DNA

### Fig. 1

The vertical lines show the location of the restriction endonuclease cleavage sites. The approximate coding regions of the cyoABCDFE genes are shown by heavy arrows. The open box shows the region carried by plasmid pTO1, b, construction of a cyo-locZY chromosomal operon fusion. The relevant restriction sites and DNA are shown. Plasmid pTO1 was integrated into the genome of E. coli strain ST7538 (polA1, AroC, thi) by homologous recombination. Linkage of amp' and cyo was confirmed by phase P1 transduction. The abbreviations used are: B, BamHI; E, EcoRI; H, HindIII; S, Sal I; T4 DNA ligase.

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### Preparation of E. coli Total RNA

E. coli total RNA was isolated essentially by the method of Alba et al. (28). Cells were harvested in the early exponential phase and suspended in 1.5 ml of a solution of 20 mM sodium acetate (pH 5.2), 0.05% (w/v) sodium dodecyl sulfate, and 1 mM EDTA. RNA was recovered by two rounds of phenol extraction at 60°C for 5 min.

### Primer Extension Analysis

Primer extension analysis was performed as described by Karrer et al. (29) with the following modifications. Fifty pmol of synthesized primer was mixed with 50 pmol of [γ-32P]ATP and 27 units of T4 polynucleotide kinase in 50 mM Tris (pH 8.0), 10 mM MgCl2, and 10 mM β-mercaptoethanol in a total volume of 100 μl. After incubation at 37°C for 20 min, DNA was precipitated with ethanol and dissolved in 50 μl of hybridization buffer (60% (v/v) formamide, 0.5 M NaCl, 1 mM EDTA, and 40 mM PIPES (pH 6.8)). The mixture was denatured by heating at 60°C for 5 min, then hybridized with 5 pmol of the primer in a reaction volume of 10 μl at 60°C for 30 min. Each primer was added to the reaction mixture after hybridization at 40°C for 30 min to a final concentration of 0.3 pmol. Primer extension reactions were performed with 10,000 cpm of 32P-labeled DNA and 100 pmol of reverse transcriptase. Incubation was at 37°C for 20 min. The products were separated by electrophoresis on a 10% denaturing polyacrylamide gel and autoradiographed. The migration rate of the primer was determined by running the primer alone under the same conditions as the samples. The migration rate of the primer alone was noted as zero on the autoradiogram.
Effect of Oxygen on cyo Gene Expression—The transcriptional activity of the cyo gene during aerobic growth was monitored by assay of the specific activity of β-galactosidase in strain ST2539, which has cyo-lacZY operon fusion (Fig. 2). The results demonstrated that cyo gene expression under aerobic conditions reached a maximum in the early exponential phase (OD560 < 0.1), and that the gene was repressed in later phase. This fact suggests that the repression of cyo gene expression resulted from the increased number of cells, or changes in the dissolved oxygen concentration in the culture. To substantiate the effect of oxygen concentration on cyo gene expression, three sets of oxygen-limited cultures were performed and their β-galactosidase activity was monitored (Fig. 3). To evaluate only the oxygen effect, cells were harvested in the early exponential phase corresponding to the peak in Fig. 2 (OD560 ≤ 0.1). About 6-fold lower expression was observed under both semiaerobic conditions (78 μM O2) and strictly anaerobic conditions (0.5 μM O2) than under aerobic conditions (240 μM O2). Thus expression of the cyo gene is clearly enhanced by a high concentration of oxygen in the medium.

Catabolite Effect on cyo Gene Expression—We examined the effects of various carbon sources on cyo gene expression. Strain ST2539 was grown aerobically with various compounds as the sole carbon source, and harvested in the early exponential phase at OD600 ≤ 0.1 (see Fig. 2), and then its β-galactosidase activity was measured. As shown in Table II, cells grown on 2% (w/v) casamino acids showed the highest enzyme expression, while cells grown on glucose showed the lowest level of about one fifth the highest level. The extents of expression, while cells grown on glucose showed the lowest level for another 60 min. The reaction products were precipitated with ethanol, solubilized in 10 μl of the loading buffer described by Maniatis et al. (17), and electrophoresed on 4% (w/v) polyacrylamide gel simultaneously with a sequence ladder generated with the same primer.

RESULTS

Effect of Oxygen on cyo Gene Expression—The transcriptional activity of the cyo gene during aerobic growth was monitored by assay of the specific activity of β-galactosidase in strain ST2539, which has cyo-lacZY operon fusion (Fig. 2). The results demonstrated that cyo gene expression under aerobic conditions reached a maximum in the early exponential phase (OD560 < 0.1), and that the gene was repressed in later phase. This fact suggests that the repression of cyo gene expression resulted from the increased number of cells, or changes in the dissolved oxygen concentration in the culture. To substantiate the effect of oxygen concentration on cyo gene expression, three sets of oxygen-limited cultures were performed and their β-galactosidase activity was monitored (Fig. 3). To evaluate only the oxygen effect, cells were harvested in the early exponential phase corresponding to the peak in Fig. 2 (OD560 ≤ 0.1). About 6-fold lower expression was observed under both semiaerobic conditions (78 μM O2) and strictly anaerobic conditions (0.5 μM O2) than under aerobic conditions (240 μM O2). Thus expression of the cyo gene is clearly enhanced by a high concentration of oxygen in the medium.

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The possibility that the reduced cyo gene expression under anaerobic conditions was due to a decreased level of intracellular cAMP was investigated. When strain ST2539 was grown anaerobically with 2.5 mM exogenous cAMP, its level of cyo gene expression was not restored to the control level (data not shown).

Nucleotide Sequence—From the above results, cyo gene expression seemed to be subject to catabolite repression and to be controlled by the oxygen concentration in the medium. Therefore, we determined the nucleotide sequence of the regulatory region of the cyo gene. The regulatory region has been mapped within the 1.7-kb SalI-SalI fragment of pHN11...
which contains the cyoA gene and its 5'-flanking region (8).

As shown in Fig. 4, there is a single open reading frame of sufficient length to encode subunit II between nucleotide position 44 and 988 (data not shown). The deduced M. of the polypeptide is 34,911 (315 residues), in good agreement with the M. of 35,000 of subunit II of the purified enzyme (4, 5). The first ATG triplet is located seven nucleotides downstream of the 3'-terminal sequence of 16 S ribosomal RNA (30).

In the upstream region of the transcription start point, we identified two potential CAP-binding sites, positions -253 to -212 for CAP1 and -81 to -60 for CAP2 (Fig. 5), that resemble the 22-bp consensus sequence, 5'-AANTGT-GANNNNGTHCANNNT-3' (31). This is consistent with the fact that the cyo operon is subject to catabolite repression. At present, it is not clear which CAP site is functional.

The cyo regulatory region contains a single region of AT-rich hyphenated dyad symmetry (positions -217 to 195) adjacent to the CAP1 site (Fig. 5). Thus this region could be a binding site of a regulatory protein for the control of expression by oxygen concentration. A computer search for other genes sharing a homologous nucleotide sequence to this region of hyphenated dyad symmetry was then carried out. Six genes having a similar region were found: the cyo gene for the cytochrome b(558-b595) complex (32), the fumA gene for succinate dehydrogenase (33), the fumC gene for fumarase (35, 36), the sdhA gene for succinate dehydrogenase (33), the lpd gene for E. coli component of 2-oxoglutarate dehydrogenase (34), and the fumC gene for fumarase.

The homologous regions of the sequences are shown in Fig. 6. The cytochrome b(558-b595) complex is an alternative terminal oxidase of the aerobic respiratory chain and is reported to be expressed in the mid- to late exponential phase (37). The other five enzymes are components of the citric acid cycle and have been shown to be repressed under anaerobic conditions (38-40).

**Transcript Analysis**—The start site of the cyo mRNA was determined by using the 5'-end of the cyo transcript by primer extension analysis. A 20-mer complementary to nucleotides 206-225 was used as a primer. As shown in Fig. 7, RNAs from the wild-type strain (ST2539) grown under aerobic conditions (lane 1) and the ΔcyA strain (ST2571) grown with 2.5 mM cAMP under aerobic conditions (lane 4) produced transcripts of identical length. Therefore, the major start site of mRNA synthesis was identified as A at position +1 (Fig. 5). Aerobically grown wild-type cells (lane 1) produced more transcripts of identical length and of the transcript than did anaerobically grown cells (lane 2). Furthermore, the absence of bands in lane 3 indicates that the synthesis of the cyo transcript required CAMP. Upstream of the start point, there are potential promoter sequences, TAAAATG (positions -12 to -6) resembling the consensus Pribnow box, TATAATG (41) and TTTACA (positions -35 to -30), homologous to the sequence TTGACA in the -35 region of many E. coli promoters. The latter sequence is most favorably positioned relative to the Pribnow box; that is, a 17 bp operon region is located between the last base in the -35 region and the first base in the Pribnow box (42).

**DISCUSSION**

In this study, we characterized the transcriptional regulation of the cyo operon. We constructed a cyo-lacZ chromosomal operon fusion and examined lacZ expression under various growth conditions, such as aerobic and anaerobic conditions and with various carbon sources.

Bromann et al. (13) showed spectroscopically that the synthesis of cytochrome o was subject to catabolite repression. Here, we showed by operon fusion that the expression of the cyo gene is subject to catabolite repression at the transcriptional level. Furthermore, to substantiate these results, we determined the nucleotide sequence of the regulatory region and found two potential CAP-binding sites. We also demonstrated qualitatively that cAMP was required for the production of the cyo transcript. All these results indicate that the cyo operon is subject to catabolite repression at the transcriptional level.
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Fig. 7. Mapping of the 5'-end of the cyo mRNA. A 32P-end-labeled oligonucleotide primer complement to base positions 206-225 in Fig. 5 was annealed to total cellular RNA and extended using deoxynucleotide triphosphates and reverse transcriptase. The DNA products were separated by electrophoresis on 4% (w/v) polyacrylamide gel simultaneously with a Sanger dideoxy chain termination ladder produced with the identical primer and the cyo sense strand products were separated by electrophoresis on 4% (w/v) polyacrylamide gel. The DNA complement. The putative Pribnow box is labeled as -10. The in vivo transcriptional level. At present, it is still unclear which CAP binding site is functional. Deletion studies on this region will answer the question.

The synthesis of cytochrome o was shown spectroscopically (11) and immunologically (12) to be derepressed by high oxygen tension. Here, we demonstrated by operon fusion that the level of cyo gene expression varied with the progression of growth phase. The cyo gene was highly expressed in the early exponential phase, and a high oxygen concentration (more than 78 μM) was shown to be required for expression of the cyo gene by the experiments using the oxygen-limited cultures. We think the repression of cyo gene expression in the late exponential and stationary phases was caused by lowered oxygen concentration. Furthermore, the amounts of transcripts were more under aerobic conditions than under anaerobic conditions, confirming the effect of oxygen concentration qualitatively. The fact that the addition of cAMP to the medium of anaerobic cultures did not restore the β-galactosidase activity indicated that the repression under anaerobic conditions was not due to depletion of intracellular cAMP. Thus, the regulatory effects of catabolites and oxygen tension on the expression of cyo gene are distinct.

At present, the promoter regions of five genes coding for enzymes that are expressed under aerobic conditions have been sequenced; these are the 5'-flanking region of sdhC (a subunit of succinate dehydrogenase), gltA (citrate synthase), lpd (E3 component of 2-oxoglutarate dehydrogenase), and fumA and fumC (fumarase). These genes encode components of the citric acid cycle and all have a region of AT-rich dyad symmetry in their 5'-flanking region like that of the cyo operon. Although the relative positions of these regions from the transcription start point differ and the hyphenated dyad symmetry is not perfect, 17 bp (sdhC/gltA, fumA) or 15 bp (lpd, fumC) of the total 23 bp are homologous. An attractive possibility is that this region is a recognition motif for an oxygen effector(s). Furthermore, there is a similar sequence in the upstream region of the cyd gene (19 of 23 bp are homologous), which codes for the alternative terminal oxidase, cytochrome b566-b567-d complex. The cyo and cyd genes are not expressed in a coordinated manner: the former is extremely oxygen-dependent, whereas the latter is moderately oxygen-dependent. However, both genes appeared to be regulated by the oxygen concentration of the medium. Additional experiments are underway to determine if the regulatory region of the cyo operon shares a common trans-effector with the genes mentioned above and if the region of dyad symmetry actually functions.

The respiratory chain of E. coli can use several compounds effectively as electron acceptors. Of the commonly available electron acceptors, such as oxygen, nitrate, and fumarate, oxygen is used preferentially under aerobic conditions. This study showed that the preferential utilization of oxygen is partially, if not entirely, ascribed to the transcriptional control. The expression of the cyd gene encoding the cytochrome b566-b567-d complex was found to be elevated from the mid-exponential to the stationary phase (37). The nar operon encoding nitrate reductase and the frd operon encoding fumarate reductase are expressed only under strictly anaerobic conditions (43, 44). The regulatory pattern of expression of the cyo gene is different from those of the other genes encoding terminal enzymes of the respiratory chain, but rather similar to those of the enzymes participating in the citric acid cycle (38, 45). When glucose is present in the medium, only the enzymes participating in the glycolytic pathway are induced and energy is obtained mainly by this pathway. But when glucose is not available and the cells are growing aerobically, nonfermentable compounds are utilized as carbon sources and enzymes of the citric acid cycle and the cytochrome b566-o complex are induced by a mechanism of oxygen induction that overcomes the regulation by cAMP. We believe that the preferential utilization systems of glucose and oxygen are independent, but contribute mutually to regulation of energy economy of the cells by restricting the formation of enzymes for the glycolytic pathway and citric acid cycle/terminal oxidase pathway.

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