Comparative transcriptomic and proteomic analysis of *Arthrobacter* sp. CGMCC 3584 responding to dissolved oxygen for cAMP production

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*Arthrobacter* sp. CGMCC 3584 is able to produce high yields of extracellular cyclic adenosine monophosphate (cAMP), which plays a vital role in the field of treatment of disease and animal food, during aerobic fermentation. However, the molecular basis of cAMP production in *Arthrobacter* species is rarely explored. Here, for the first time, we report the comparative transcriptomic and proteomic study of *Arthrobacter* cells to elucidate the higher productivity of cAMP under high oxygen supply. We finally obtained 14.1% and 19.3% of the *Arthrobacter* genome genes which were up-regulated and down-regulated notably, respectively, with high oxygen supply, and identified 54 differently expressed proteins. Our results revealed that high oxygen supply had two major effects on metabolism: inhibition of glycolysis, pyruvate metabolism, nitrogen metabolism, and amino acid metabolism (histidine, branched-chain amino acids and glutamate metabolism); enhancement of the tricarboxylic acid cycle and purine metabolism. We also found that regulation of adenylate cyclase and phosphodiesterase was not significant under high oxygen supply, suggesting efficient cAMP export might be important in cAMP production. These findings may contribute to further understanding of capacities of *Arthrobacter* species and would be highly useful in genetic regulation for desirable production.

Cyclic adenosine monophosphate (cAMP) is an important compound which exists in many living cells, and participates in the regulation of physiological actions, such as cell proliferation and differentiation, hormones synthesis and secretion, membrane protein activity, nervous activity, gene expression and so on\(^1,3\). Based on its functions, cAMP is used in pharmaceuticals and as feed additives. The industrial production of cAMP through microorganism attracts attention of researchers for low cost and environmental friendliness. The microbes, including *Microbacterium*, *Arthrobacter*, *Brevibacterium liquefaciens*, *Corynebacterium roseoparaffineus*, *Corynebacterium murisepticum*, were reported to be able to produce cAMP\(^4,5\).

Our previous studies have demonstrated that a strain, *Arthrobacter* sp. CGMCC 3584, was capable of producing high yields of cAMP in culture media, through optimization of culture conditions and metabolic regulation on the fermentation process\(^6-8\). *Arthrobacter* species are among the most common aerobic cultivable bacteria and exist in the nature widely, mainly in soil and in some extreme environments\(^9,10\). Their remarkable tolerance to various stresses contributes to the application of *Arthrobacter* species in contaminant degradation in complex and volatile environments\(^11\). Most genome and transcriptome researches of this genus focus on the genetic basis of the biodegradation and survival capacities, and available microarray data are rarely thus far. In the present study, DNA microarray-based transcriptomic analysis and two-dimensional (2D) gel electrophoresis technology

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and mass spectrum (MS) based proteomic analysis of Arthrobacter cells cultured under various oxygen supply conditions was conducted to dissect the molecular regulatory mechanism of cAMP production.

It has been reported that oxygen supply, which could influence nutrients uptake, cell metabolism and product yield, plays an important role in aerobic fermentation12. Oxygen can affect the expression patterns of genes involved in various cell functions including carbon metabolism, iron uptake and stress response13. Many transcriptomic studies were conducted to gain insight into the mechanism of fermentative lifestyle evolution under different conditions of oxygen availability. For instance, a microarray study on Kluyveromyces lactis showed that the availability of oxygen determined the fermentation pattern, with increasing the glucose metabolism and reducing fluxes in the pentose phosphate pathway under oxygen-limited conditions14. Another DNA microarray analysis of the full transcriptome of the Bordetella pertussis bacterium revealed that oxygen limitation during cultivation had a fully reversible effect on gene expression. Microarray analysis was also used to investigate the global gene expression of Lactococcus lactis subsp. lactis and results indicated that trehalose and GTP were implicated in bacterial adaptation to oxidative stress15. Our previous experiments confirmed dissolved oxygen (DO) level definitely affected cAMP production during fermentation16. However, the intrinsic relationship between oxygen supply and cAMP biosynthesis is still not so clear. Thus far, no transcriptomic and proteomic analysis of cAMP-producing bacterial cells has been reported. In this work, we try to comprehensively explore the underlying mechanisms of oxygen supply on cAMP biosynthesis, which will help to better understand the metabolic regulation mechanism of Arthrobacter species and provide valuable information for further improvement of cAMP production.

Results

Oxygen supply and cAMP production. In the 5-L fermenters, two batches of cAMP fermentation were carried out with agitation set at 150 and 350 r/min to construct low oxygen supply and high oxygen supply conditions, respectively. As shown in Fig. 1, the DO level showed a sharp drop in the initial phase of fermentation both in low and high oxygen supply. The DO concentration reduced to nearly zero at 3 h and began to rise until 36 h with 150 r/min agitation, while the DO was at a much higher level with 350 r/min agitation. After 66 h fermentation, the consumption of glucose under high oxygen supply was almost twice as under low oxygen supply. The highest DCW values and cAMP yields reached 4.91 ± 0.05 g/L and 2.34 ± 0.21 g/L, respectively, under low oxygen supply, and 10.94 ± 0.41 g/L and 11.17 ± 0.81 g/L, respectively, under high oxygen supply. The final cAMP yield on DCW reached 0.57 ± 0.027 g/g DCW and 1.02 ± 0.03 g/g DCW under low and high oxygen supply respectively. This result showed that the oxygen supply condition had a greater influence on cAMP production than cell growth.

Figure 1. The time courses of DCW (a) and cAMP (b), and DO (c) and glucose (d) in the 5-L bioreactor fermentation with low oxygen supply (L) and high oxygen supply (H).
respectively. Among these differentially expressed genes, 176, 189, 237 and 178 genes were up-regulated (ratio \( \frac{\text{high}}{\text{low}} \geq 2 \)) with high oxygen supply for 12 h, 24 h, 36 h and 48 h samples, respectively. Among these differentially expressed genes, 176, 189, 237 and 178 genes were up-regulated (ratio \( \frac{\text{high}}{\text{low}} \geq 2 \)) with high oxygen supply for 12 h, 24 h, 36 h and 48 h samples, respectively. While compared to the low oxygen supply condition, 174, 318, 434 and 268 genes were down-regulated (ratio \( \frac{\text{high}}{\text{low}} \leq 0.5 \)) at 12 h, 24 h, 36 h and 48 h respectively. As shown in Fig. 2, a total of 574 genes, representing 14.1% of the \textit{Arthrobacter} genome genes, exhibited up-regulation of mRNA expression level and 788 genes, representing 19.3% of the \textit{Arthrobacter} genome genes, were down-regulated significantly with high oxygen supply. Regarding the differentially expressed genes shared by the samples of four time points, 11 were increased in relative expression level (Fig. 2a) and 13 were decreased in relative expression level (Fig. 2b).

These differently expressed genes fell into major Clusters of Orthologous Groups (COG) categories (shown in Fig. 3, based on the NCBI-COG database). The largest group with altered transcriptional levels belonged to the group involved in carbohydrate transport and metabolism (11.9% up-regulated and 15.6% down-regulated). Other large functional groups of differentially expressed genes changed significantly included amino acid transport and metabolism (14.8% up-regulated and 12.1% down-regulated), inorganic ion transport and metabolism (10.9% up-regulated and 7.6% down-regulated), and transcription (9.0% up-regulated and 9.1% down-regulated). And 5.3% genes were annotated as function unknown. The genes associated with RNA processing and modification, chromatin structure and dynamics, nuclear structure, cytoskeleton and extracellular structures did not show any significant changes with high oxygen supply at all four time points.

**General changes on proteomic level.** Cell samples of \textit{Arthrobacter} sp. CGMCC 3584 were collected after 12 h, 24 h, 36 h and 48 h fermentation under different oxygen supply conditions for microarray analysis. The oxygen supply condition appeared to have a significant effect on global transcript levels in \textit{Arthrobacter} sp. CGMCC 3584 for cAMP fermentation. As shown in Supplementary Table S1, 350, 507, 671 and 446 genes were identified to differentially express using an empirical 2-fold criterion, for 12 h, 24 h, 36 h and 48 h samples, respectively. Among these differentially expressed genes, 176, 189, 237 and 178 genes were up-regulated (ratio \( \frac{\text{high}}{\text{low}} \geq 2 \)) with high oxygen supply for 12 h, 24 h, 36 h and 48 h samples, respectively. Among these differentially expressed genes, 176, 189, 237 and 178 genes were up-regulated (ratio \( \frac{\text{high}}{\text{low}} \geq 2 \)) with high oxygen supply for 12 h, 24 h, 36 h and 48 h samples, respectively. While compared to the low oxygen supply condition, 174, 318, 434 and 268 genes were down-regulated (ratio \( \frac{\text{high}}{\text{low}} \leq 0.5 \)) at 12 h, 24 h, 36 h and 48 h respectively. As shown in Fig. 2, a total of 574 genes, representing 14.1% of the \textit{Arthrobacter} genome genes, exhibited up-regulation of mRNA expression level and 788 genes, representing 19.3% of the \textit{Arthrobacter} genome genes, were down-regulated significantly with high oxygen supply. Regarding the differentially expressed genes shared by the samples of four time points, 11 were increased in relative expression level (Fig. 2a) and 13 were decreased in relative expression level (Fig. 2b).

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**General changes on proteomic level.** Cell samples of \textit{Arthrobacter} sp. CGMCC 3584 were collected after 12 h and 36 h fermentation under different oxygen supply conditions for 2D gel separation. The 2D gel images were shown in Fig. 4. Protein expression level ratios (high oxygen supply/low oxygen supply) \( \geq 2.5 \) or \( \leq 0.4 \) \( (p < 0.05) \) were considered to be significantly regulated. As a result, a total of 67 differentially expressed protein spots showed changes of significance in their intensities under different oxygen supply (listed in Supplementary Table S2). For 12 h samples, 11 protein spots were significantly up-regulated with high oxygen supply (ratio \( \frac{\text{high}}{\text{low}} \geq 2.5 \)), whereas 5 spots were down-regulated (ratio \( \frac{\text{high}}{\text{low}} \leq 0.4 \)). For 36 h samples, 38 protein spots were significantly up-regulated with high oxygen supply (ratio \( \frac{\text{high}}{\text{low}} \geq 2.5 \)), whereas 22 spots were down-regulated (ratio \( \frac{\text{high}}{\text{low}} \leq 0.4 \)). These protein spots were further analyzed by MALDI-TOF/TOF-MS and 54 spots among them were successfully identified (listed in Table 1). Among these 54 spots, twelve different protein spots were identified to represent six gene products, including succinyl-CoA synthetase subunit beta (ID: 8229, 8515), molecular chaperone DnaK (ID: 1005, 1029), elongation factor Ts (ID: 1208, 4218), threonyl-tRNA synthetase (ID: 4819, 3802), elongation factor Tu (ID: 8227, 8501), and phosphomannomutase (ID: 7610, 8078). So we finally obtained 48 different proteins.

The distribution of the differentially expressed proteins over the different COG categories was exhibited in Fig. 5. Among these successfully identified spots, 32, 11 and 10 proteins belonged to metabolism, information storage and processing, and cellular processes and signaling, respectively. And 3 proteins were poorly characterized. According to the COG categories, the up-regulated proteins under high oxygen supply were mainly involved in carbohydrate transport and metabolism, energy production and conversion, and posttranslational modification, protein turnover, chaperones. The majority of the down-regulated proteins were classified as carbohydrate transport and metabolism, and translation, ribosomal structure and biogenesis.

**Quantitative real-time PCR (qRT-PCR) validation of the genes transcription levels.** In order to validate the microarray data, we used qRT-PCR to further investigate the mRNA expression of five selected genes for 12 h and 36 h samples, including ribose-phosphate pyrophosphokinase (gismo_orf1245), pyruvate
kinase (gismo_orf2621), fructose-bisphosphate aldolase (gismo_orf2812), succinyl-CoA synthetase subunit beta (gismo_orf3726) and phosphoglycerate kinase (gismo_orf3896). As shown in Table 2, the mRNA ratios obtained by qRT-PCR were mostly in good agreement with the fold changes from the microarray data analysis, except the expressions of fructose-bisphosphate aldolase (12 h samples) and phosphoglycerate kinase (36 h samples) showed some inconsistency between qRT-PCR and microarray results. In addition, the relevant protein fold changes were also shown in Table 2. The protein abundance of ribose-phosphate pyrophosphokinase (spot 211), pyruvate kinase (spot 3805), fructose-bisphosphate aldolase (spot 7103) and succinyl-CoA synthetase subunit beta (spot 8229 and 8515) exhibited the similar fold changes with the relevant qRT-PCR results at 12 h or 36 h with high oxygen supply. And the results of qRT-PCR and protein analysis of phosphoglycerate kinase (spot 7511) were inconsistent for both 12 h and 36 h samples.

Discussion

Limited information is available on the molecular mechanism of cAMP-producing fermentation. Here, we reported a comprehensive transcriptome and proteome study to characterize the gene and protein expression profiles of a cAMP-producing strain, *Arthrobacter* sp. CGMCC 3584, responding to DO level. The important genes and proteins with significantly differential expressions, related to the central carbon metabolic network, were shown in Fig. 6.
The phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) is a major mechanism for uptake of carbohydrates in bacteria, where PEP is used as an energy source and phosphoryl donor. The PTS carries out its catalytic function in sugar transport and phosphorylation by two general components, including enzyme I (EI) and histidine phosphocarrier protein (HPr), and of membrane-bound sugar specific permeases (enzymes II). It is observed that the PTS can regulate the cAMP concentration and adenylate cyclase activity. For example, the addition of glucose and other PTS carbohydrates inhibits cAMP synthesis in E. coli17. Furthermore, the PTS not only controls carbohydrate uptake and metabolism but also influences the utilization of nitrogen and phosphorus. In our study, some proteins involved in glucose utilization were down-regulated under high oxygen supply. One carbohydrate uptake protein, PEP-protein phosphotransferase (spot 7703), exhibited a significant reduction at 12 h as well as its mRNA level (gismo_orf2319) with high oxygen supply. Meanwhile, microarray results indicated that genes encoding HPr family protein (gismo_orf1682), maltose/glucose porter, IIABC component (gismo_orf1998), beta-glucoside-specific, IIABC component (gismo_orf502), and mannitol-specific IIC component (gismo_orf2322) which involved in PTS system were down-regulated significantly.

Three proteins involved in glycolysis, including phosphoglycerate kinase (spot 7511), pyruvate kinase (spot 3805), and triosephosphate isomerase (spot 1405) were also down-regulated significantly with high oxygen supply at 12 h or 36 h, and their transcriptional levels were decreased significantly as well (gismo_orf3896, gismo_orf2621 and gismo_orf3895, respectively). The microarray results also showed that gene expression levels of 6-phospho-beta-glucosidase (gismo_orf501), 6-phosphofructokinase (gismo_orf202) and glyceraldehyde-3-phosphate dehydrogenase (gismo_orf3897) which involved in glycolysis were reduced significantly under high oxygen supply. In addition, the pathways of pyruvate metabolism tended to be restrained under high oxygen supply. The genes encoding PEP carboxylase (gismo_orf2894), 2-isopropylmalate synthase
| Spot no. | NCBI accession no. | Protein name             | Species                        | Proteome fold changes | Experimental mass (kDa) and pI | Score |
|---------|-------------------|--------------------------|--------------------------------|-----------------------|--------------------------------|-------|
| 113     | gi|72163016 | 50 S ribosomal protein L17 | Thermobifida fusca YX       | 2.78 5.57E-06 43.78/4.82 | 58   |
| 204     | gi|50955503 | succinyl-CoA synthetase subunit alpha | Leifsonia xyli subsp. xyli str. CTC057 | 0.38 1.71E-02 52.87/5.29 | 68   |
| 211     | gi|220912059 | ribose-phosphate pyrophosphokinase | Arthrobacter chlorophenolicus A6 | 3.79 2.93E-04 52.19/4.7 | 74   |
| 405     | gi|325962845 | protein RecA | Arthrobacter phenanthrenivorans Sphe3 | 0.26 1.35E-03 64.93/4.58 | 508  |
| 424     | gi|444307928 | threonine synthase | Arthrobacter sp. SJCon | 2.57 4.30E-04 63.44/4.76 | 154  |
| 522     | gi|325962559 | serine hydroxymethyltransferase | Arthrobacter phenanthrenivorans Sphe3 | 3.38 1.88E-07 76.21/5.18 | 247  |
| 602     | gi|119961574 | 6-phosphogluconate dehydrogenase | Arthrobacter aurecens TC1 | 0.34 7.31E-04 83.35/4.99 | 186  |
| 705     | gi|220912286 | CTP synthetase | Arthrobacter chlorophenolicus A6 | 2.99 6.63E-07 90.53/5.3 | 399  |
| 1002    | gi|116670901 | ribonucleotide-diphosphate reductase subunit beta | Arthrobacter sp. FB24 | 3.94 1.22E-02 23.42/5.49 | 58   |
| 1004    | gi|220910796 | peptidyl-prolyl isomerase | Arthrobacter chlorophenolicus A6 | 4.79 1.75E-06 25.6/5.56 | 252  |
| 1005    | gi|3023655 | Chaperone protein DnaK | Arthrobacter chlorophenolicus A6 | 11.27 4.72E-04 25.6/5.56 | 252  |
| 1029    | gi|116672350 | molecular chaperone DnaK | Arthrobacter chlorophenolicus A6 | 3.94 2.02E-03 22.84/5.59 | 252  |
| 1206    | gi|220913188 | winged helix family two component transcriptional regulator | Arthrobacter chlorophenolicus A6 | 3.59 3.09E-04 53.25/5.59 | 80   |
| 1208    | gi|220912155 | elongation factor Ts | Arthrobacter chlorophenolicus A6 | 4.37 1.18E-04 53.01/5.5 | 259  |
| 1405    | gi|325963192 | triosephosphate isomerase | Arthrobacter phenanthrenivorans Sphe3 | 0.07 4.37E-03 66.41/5.5 | 81   |
| 1421    | gi|116696487 | sugar ABC transporter, ATP-binding protein | Arthrobacter aurecens TC1 | 3.12 5.05E-04 69.45/5.09 | 107  |
| 1708    | gi|11669060 | NAD synthetase | Arthrobacter sp. FB24 | 0.22 5.15E-03 93.34/5.48 | 146  |
| 1715    | gi|119950307 | dihydroxy-acid dehydratase | Arthrobacter aurecens TC1 | 0.29 9.31E-03 93.26/5.55 | 112  |
| 1718    | gi|119964210 | aspartyl-tRNA synthetase | Arthrobacter aurecens TC1 | 0.29 4.78E-04 93.61/5.6 | 64   |
| 1803    | gi|359777979 | catalase | Arthrobacter globiformis NBRC 12137 | 0.36 1.40E-02 107.08/5.58 | 91   |
| 1828    | gi|220911112 | ATPase AAA | Arthrobacter phenanthrenivorans Sphe3 | 2.35 4.62E-02 62.22 4.39E-05 106.76/4.98 | 495  |
| 2505    | gi|325963975 | glutamate-1-semialdehyde 2,1-aminomutase | Arthrobacter phenanthrenivorans Sphe3 | 2.80 6.70E-03 75.36/5.77 | 241  |
| 3310    | gi|116670653 | FeS assembly ATPase SafC | Arthrobacter sp. FB24 | 0.07 2.43E-05 62.6/5.94 | 172  |
| 3606    | gi|119964256 | glycogen synthase | Arthrobacter aurecens TC1 | 0.24 8.44E-05 79.21/5.89 | 239  |
| 3802    | gi|116670864 | threonyl-tRNA synthetase | Arthrobacter sp. FB24 | 0.28 1.24E-03 96.06/5.83 | 138  |
| 3805    | gi|220912455 | pyruvate kinase | Arthrobacter chlorophenolicus A6 | 0.33 3.49E-03 95.81/5.89 | 118  |
| 4218    | gi|220912155 | elongation factor Ts | Arthrobacter chlorophenolicus A6 | 0.35 6.53E-05 51.32/5.87 | 219  |
| 4819    | gi|220912797 | threonyl-tRNA synthetase | Arthrobacter chlorophenolicus A6 | 3.29 4.54E-05 101.65/6.01 | 189  |
| 5005    | gi|325962142 | uracil phosphoribosyltransferase | Arthrobacter phenanthrenivorans Sphe3 | 0.36 9.82E-05 38.3/6.2 | 294  |
| 5711    | gi|325964075 | chaperonin GroL | Arthrobacter phenanthrenivorans Sphe3 | 3.56 1.20E-04 90.28/6.15 | 713  |
| 6109    | gi|220912628 | short chain dehydrogenase | Arthrobacter chlorophenolicus A6 | 15.97 5.33E-06 41.96/6.2 | 329  |
| 6850    | gi|325963015 | aconitase | Arthrobacter phenanthrenivorans Sphe3 | 2.77 1.46E-06 106.48/6.08 | 723  |
| 7003    | gi|325963765 | ketol-acid reductoisomerase | Arthrobacter phenanthrenivorans Sphe3 | 0.21 5.89E-07 37.9/6.38 | 413  |
| 7103    | gi|116669084 | fructose-bisphosphate aldolase | Arthrobacter sp. FB24 | 3.72 2.21E-05 47.01/6.39 | 305  |

Continued
| Spot no. | NCBI accession no. | Protein name | Species | Proteome fold changes | Experimental mass (kDa) and pI | Score |
|----------|--------------------|--------------|---------|-----------------------|-------------------------------|-------|
| 7115     | gi|325964935       | phosphoribosylanthranilate isomerase | Arthrobacter phenanthrenivorans Sphe3 | 17.86 9.03E-08 | 41.64/6.32 160 |
| 7118     | gi|325963063       | response regulator with antiterminator output domain | Arthrobacter phenanthrenivorans Sphe3 | 7.38 1.21E-04 | 35.6/6.47 93 |
| 7210     | gi|116668714       | inorganic diphosphatase | Arthrobacter sp. FB24 | 2.50 6.31E-03 | 49.57/6.46 109 |
| 7404     | gi|220913400       | DNA-directed RNA polymerase subunit alpha | Arthrobacter chlorophenolicus A6 | 0.29 7.77E-03 | 64.16/6.39 400 |
| 7505     | gi|325962588       | enolase | Arthrobacter phenanthrenivorans Sphe3 | 2.60 8.29E-05 | 70.14/6.4 760 |
| 7511     | gi|325963191       | phosphoglycerate kinase | Arthrobacter phenanthrenivorans Sphe3 | 0.30 9.03E-04 | 72.17/6.53 320 |
| 7605     | gi|325962719       | aspartyl/glutamyl-tRNA amidotransferase subunit A | Arthrobacter phenanthrenivorans Sphe3 | 0.37 1.20E-02 | 81.67/6.38 623 |
| 7610     | gi|220911593       | phosphomannomutase | Arthrobacter chlorophenolicus A6 | 0.32 3.72E-03 | 83.91/6.42 291 |
| 7703     | gi|116672548       | phosphoenolpyruvate-protein phosphotransferase | Arthrobacter sp. FB24 | 0.23 2.60E-02 | 87.25/6.42 219 |
| 7704     | gi|220914230       | glucose-methanol-choline oxidoreductase | Arthrobacter chlorophenolicus A6 | 3.77 1.62E-04 | 95.29/6.43 80 |
| 7805     | gi|119961649       | 2-oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyltransferase | Arthrobacter aurescens TC1 | 3.11 3.31E-05 | 100.9/6.37 445 |
| 7813     | gi|116670612       | 30 S ribosomal protein S1 | Arthrobacter sp. FB24 | 4.09 3.64E-04 | 95.41/6.46 434 |
| 8078     | gi|220911593       | phosphomannomutase | Arthrobacter chlorophenolicus A6 | 5.20 1.19E-03 | 31.22/6.45 606 |
| 8227     | gi|116671525       | elongation factor Tu | Arthrobacter sp. FB24 | 6.29 2.15E-03 | 50.02/6.72 142 |
| 8229     | gi|116669380       | succinyl-CoA synthetase subunit beta | Arthrobacter sp. FB24 | 6.56 1.62E-04 | 49.57/6.78 227 |
| 8302     | gi|220911115       | dihydroxyacetone kinase subunit DhaK | Arthrobacter chlorophenolicus A6 | 4.18 1.09E-03 | 62.6/6.61 662 |
| 8501     | gi|116671525       | elongation factor Tu | Arthrobacter sp. FB24 | 0.10 4.81E-06 | 72.51/6.58 224 |
| 8514     | gi|444304706       | FoF1 ATP synthase subunit beta | Arthrobacter sp. SJCon | 6.67 6.23E-05 | 72.83/6.49 461 |
| 8515     | gi|116669380       | succinyl-CoA synthetase subunit beta | Arthrobacter sp. FB24 | 10.20 1.84E-04 | 76.21/6.52 292 |
| 9402     | gi|116670134       | cell division protein FtsZ | Arthrobacter sp. FB24 | 5.01 5.66E-05 | 68.1/6.79 78 |

**Table 1.** Identified differentially expressed proteins responding to different oxygen supply in *Arthrobacter* sp. CGMCC 3584.

**Figure 5.** Function classifications of differentially expressed proteins (fold changes of at least 2.5) at 12 h (a) and 36 h (b). For abbreviations, see the legend to Fig. 3.
Bacillus subtilis, and increasing cellular PRPP pool resulted in induction of gene expression in the purine biosynthetic pathway. Here, we observed that the ribose-phosphate fermentation of Torulopsis glabrata in this strain, and cAMP synthesis was mainly via the salvage route. In the de novo route, phosphoribosyl-cAMP could be produced via de novo biosynthesis or salvage biosynthesis from additional hypoxanthine (HX) were supplied for purine biosynthesis, which benefited cAMP production. Additionally, several enzymes associated with the active TCA cycle. Meanwhile, the gene encoding PEP carboxykinase (gismo_orf3667), which played an important role in balancing the metabolite pool between the TCA cycle and glycolysis by catalyzing anaplerotic reaction from oxaloacetate (OAA) to PEP, was significantly up-regulated at 36 h. These up-regulated proteins and genes mentioned above suggested that relatively high activity of the TCA cycle provided sufficient ATP to maintain cell growth and promote cAMP production.

Promotion of 5-phosphoribosyl 1-pyrophosphate (PRPP) synthesis and purine metabolism is one of the reasons that high oxygen supply enhanced cAMP production. Two PRPP synthesis related enzymes in PP pathway, ribulose-5-phosphate 3-epimerase and ribose-phosphate pyrophosphokinase and were considered to be overexpressed under high oxygen supply. Ribulose-5-phosphate 3-epimerase catalyzed the interconversion of ribulose-5-phosphate and xylulose-5-phosphate and its coding gene (gismo_orf2643) was obviously up-regulated at 36 h. Ribose-phosphate pyrophosphokinase is known to be implicated in the pyrophosphorylation of ribose-5-phosphate to PRPP as a regulatory enzyme in purine biosynthesis, and PRPP produced by this enzyme is of importance for purine biosynthetic pathway. For instance, PRPP is one important effector molecule in the biosynthesis of inosine monophosphate (IMP) in Bacillus subtilis, and increasing cellular PRPP pool resulted in induction of gene expression in the purine biosynthetic pathway. Here, we observed that the ribose-phosphate pyrophosphokinase (spot 211, gismo_orf1245) increased in its abundance with high oxygen supply, which was consistent with its mRNA change at 36 h. Higher expression levels of the two enzymes implied that more PRPP were supplied for purine biosynthesis, which benefited cAMP production. Additionally, several enzymes associated with purine metabolism were more abundant under high oxygen supply. Our previous studies showed that cAMP could be produced via de novo biosynthesis or salvage biosynthesis from additional hypoxanthine (HX) in this strain, and cAMP synthesis was mainly via the salvage route. In the de novo route, phosphoribosyl-formylglycinamidine synthase (PFAS) catalyzes the fourth step of IMP synthesis. Our chip data displayed three genes (gismo_orf2828, gismo_orf2829 and gismo_orf2831) encoding PFAS II, PFAS I and purS respectively, were induced significantly with high DO level at least at one time point. In the salvage route, HX and PRPP are catalyzed to IMP by hypoxanthine phosphoribosyltransferase. Our study showed that hypoxanthine phosphoribosyltransferase (gismo_orf556) was up-regulated at the mRNA level at 36 h. Along with this, the adenylosuccinate synthetase (gismo_orf2817) which involved in adenine ribonucleotide biosynthesis showed positive change in its mRNA level at 36 h with high oxygen supply. Meanwhile, the transcriptome data indicated that genes encoding citrate synthase (gismo_orf3452) and isocitrate dehydrogenase (gismo_orf1376) which were important rate-limiting enzymes in TCA cycle were up-regulated significantly at 36 h, as well as the genes encoding the succinyl-CoA synthetase (gismo_orf3725 and gismo_orf3726). And there were no genes showing obvious reduction in their mRNA levels in the TCA cycle. Three genes which involved in encoding pyruvate dehydrogenase complex (gismo_orf2295, gismo_orf4417 and gismo_orf3439) were induced significantly, indicating that the biosynthesis of acetyl-CoA from pyruvate might be promoted with high oxygen supply. The enhanced formation of acetyl-CoA was associated with the active TCA cycle. Meanwhile, the gene encoding PEP carboxykinase (gismo_orf3667), which played an important role in balancing the metabolite pool between the TCA cycle and glycolysis by catalyzing anaplerotic reaction from oxaloacetate (OAA) to PEP, was significantly up-regulated at 36 h. These up-regulated proteins and genes mentioned above suggested that relatively high activity of the TCA cycle provided sufficient ATP to maintain cell growth and promote cAMP production.

| Gene_ID       | Protein spot no. | Gene description                        | Microarray fold change | Protein fold change | qRT-PCR fold change |
|---------------|------------------|-----------------------------------------|------------------------|---------------------|---------------------|
| gismo_orf1245 | 211              | ribose-phosphate pyrophosphokinase      | 1.69                   | 2.09                | 0.97                | 3.79                | 2.48 | 3.48 |
| gismo_orf2621 | 3805             | pyruvate kinase                         | 0.44                   | 0.52                | 0.49                | 0.33                | 0.56 | 0.60 |
| gismo_orf2812 | 7103             | fructose-bisphosphate aldolase          | 0.94                   | 0.66                | 1.23                | 3.72                | 1.38 | 0.92 |
| gismo_orf3726 | 8229 and 8515    | succinyl-CoA synthetase subunit beta     | 1.19                   | 2.90                | 2.33 and 1.03      | 10.20 and 6.56      | 1.35 | 4.82 |
| gismo_orf3896 | 7511             | phosphoglycerate kinase                 | 0.43                   | 0.40                | 1.09                | 0.30                | 0.49 | 1.01 |

Table 2. Correlation between microarray and qRT-PCR results.
phosphatase (gismo_orf998) which catalyzing the third and penultimate steps in histidine biosynthesis respectively, showed notable decreases in their mRNA levels at 24 h or 48 h. Meanwhile, the genes encoding urocanate hydratase (gismo_orf2746) and imidazolonepropionase (gismo_orf1167) which catalyzed the second and third steps of histidine degradation, respectively, were down-regulated significantly at 24 h or 48 h. Since purine nucleotides and the amino acid histidine both used PRPP as the biosynthetic precursors 24, the remarkable decrease of four genes involved in histidine metabolism indicated more substrates were supplied for cAMP synthesis. The branched-chain amino acids are important for the synthesis of proteins and membranes probably representing cell growth, and their biosynthesis is regulated by some global transcriptional regulators of cellular metabolism, including carbon metabolism and nitrogen metabolism25. In most Gram-positive bacteria, the genes for the first two enzymes of the branched-chain amino acid biosynthesis (acetolactate synthase and ketol-acid reductoisomerase) belong to the same operon. In our study, the genes encoding acetolactate synthase (gismo_orf871 and gismo_orf872) and ketol-acid reductoisomerase (gismo_orf870) showed reductions at mRNA levels. Meanwhile, 2-isopropylmalate synthase (gismo_orf1668) and 3-isopropylmalate dehydrogenase (gismo_orf865) which participated in biosynthesis of L-leucine were both strongly repressed in their expression levels. Methylmalonate-semialdehyde dehydrogenase (gismo_orf2788) which related to branched-chain amino acids degradation was down-regulated as well. One gene encoding ABC branched-chain amino acid transporter (gismo_orf1905) was also found to be repressed significantly. Glutamate is one of the amino acids generated by the breakdown of the branched-chain amino acids. Eight genes involved in glutamate metabolism showed reduced expression under high oxygen supply, including L-glutamine synthetase (gismo_orf3527), glutamate synthase (gismo_orf2622), glutamate synthase (NADH) small subunit (gismo_orf2623), L-glutaminase (gismo_orf3628), N-acetylglutamate synthase (gismo_orf518), gamma-glutamyltransferase (gismo_orf4371), and glutamate-cysteine ligase (gismo_orf1593 and gismo_orf2385). The lower activity of glutamine synthetase under high oxygen supply was probably due to the higher yield of cAMP, because expression of glutamine synthetase showed a decreased trend for glucose-grown cells when cAMP was present26.

Glutamine synthetase which catalyzes the condensation of glutamate and ammonia to form glutamine and glutamate synthase which catalyzes the synthesis of glutamate from 2-oxoglutarate and glutamine also play essential roles in nitrogen metabolism. Nitrogen is necessary for the production of amino acids, nucleotides, amino sugars, NAD, and so on. Ammonium is a preferential nitrogen source, as it can be assimilated directly into glutamine and glutamate27. And glutamine or glutamate act as sensed amino acids to indicate an internal nitrogen level28. Additionally, three genes encoding the enzymes which participate in nitrogen metabolism, including assimilatory nitrite reductase (NAD(P)H) (gismo_orf1222 and gismo_orf1223) and assimilatory nitrate reductase (NADH) (gismo_orf1215), were repressed significantly as well. In our study, high oxygen supply also displayed negative effect on ammonium transporter. Ammonium transport proteins are a class of integral membrane proteins that specifically shuttle ammonium across biological membranes. In bacteria, ammonium transport proteins are used to scavenge NH$_4^+$/NH$_3$ from their environment for uptake and assimilation of nitrogen, and they facilitate ammonium uptake when the extracellular ammonium levels are low29,30. Four genes (gismo_orf8826, gismo_orf8825, gismo_orf8821, and gismo_orf8820) were repressed significantly in our study.
transport protein were negatively regulated responding to Lrp in authors are known to regulate multiple cellular metabolisms globally (Lrp) or locally (AsnC). Members of the Lrp/AsnC family proteins which might be limited to bacteria and archaea. Lrp/AsnC family transcriptional regulators are sometimes referred to as the Lrp/AsnC (leucine-responsive regulatory protein/asparagine synthase C products) family. Proteins of this family which are limited to bacteria and archaea. Lrp/AsnC family transcriptional regulators are known to regulate multiple cellular metabolisms globally (Lrp) or locally (AsnC). Members of the Lrp/AsnC family were reported to negatively regulate expression of branched-chain amino acid transport in E. coli or and B. subtilis. For example, genes encoding a putative glutaminase and a putative amino acid/amino transport protein were negatively regulated responding to Lrp. Therefore the repression of glutamate and branched-chain amino acid metabolism might be associated with induced AsnC family transcriptional regulatory functions in our study. Lrp proteins are involved in the regulation of the expression of enzymes. The PadR family is related to the bacterial archaeal MarR family of transcriptional regulators which regulate a variety of biological functions, including resistance to multiple antibiotics and other toxic chemicals, adaptation to different environments and the expression of virulence factors. In our study, two genes encoding PadR family transcriptional regulator (gismo_orf886) and MarR family transcriptional regulator (gismo_orf886) both displayed significant down-regulation when cAMP achieved higher yield.

Some research suggested that the morphology of Arthrobacter cells was related to the cAMP levels of the cells, such as the cAMP levels in the rod stage cells are greater than in the spherical stage cells, or the extracellular cAMP levels increased significantly at the onset of stationary phase. Biotin was reported to benefit the normal morphogenetic expression of Arthrobacter species. The gene (gismo_orf4076) encoding biotin synthase which catalyzes the formation of biotin was up-regulated under high oxygen supply, suggesting that higher yields of cAMP might be related to normal morphogenesis in this strain. The control of cAMP levels was considered to associate with cAMP excretion, cAMP hydrolysis by phosphodiesterase, and cAMP formation by adenylate cyclase (AC). Since AC and phosphodiesterase exhibited nonsignificant changes under high oxygen supply, we hypothesized that massive accumulation of extracellular cAMP was due to efficient cAMP export in our strain. The mechanism of cAMP exit has been ascribed to an energy-dependent extrusion pump. For example, MRP4, 5, and 8, which were members of the multidrug-related protein (MRP) family, had been shown to their ability to transport cAMP. One gene, abcB3, which was one of the ABC transporters, could export cAMP in Dictyostelium discoideum. AC toxin is known as a novel protein toxin, which is discovered by the presence of AC catalytic activity in commercial pertussis vaccines. The overproducing cAMP from this toxin is able to disorganize cellular signaling processes and cause cell death. Thus we supposed that the exporter of cAMP might belong to a kind of drug efflux transporters. The microarray results showed that one gene (gismo_orf371) encoding Major Facilitator Superfamily transporter was induced obviously. However, the mechanism of cAMP secretion in Arthrobacter species remains poorly elucidated and the functions of multidrug transporters and ABC transporters of Arthrobacter species were largely unknown. Therefore further investigations need to be done in order to test the hypothesis and to explain the cAMP export system in this strain.

The expression profiles of proteins and mRNAs are hypothesized to be strongly correlated based on the central dogma of molecular biology. However, the correlation between mRNA and protein abundances has been reported to be poor in most reports. The discrepancy between mRNA and protein levels may be due to stabilities of RNA and protein, functional categories of a given gene/protein, protein regulation by post-translational modification, post-transcriptional regulation of protein amounts, experimental measurement errors, deficiency of mathematical analysis, and so on. Some researches exhibited that expression levels of proteins and mRNAs correspond reasonably well on the whole, except some proteins and genes showing discordance. Several studies showed that only 20–40% of the changes in protein levels were attributed to variable mRNA levels. Moreover, many studies showed only a weak correlation or no correlation between mRNA and protein abundances. In our study, 25 out of 54 successfully identified proteins exhibited the expression pattern disagreement between their abundances and mRNA levels. For instance, the protein abundance of 6-phosphogluconate dehydrogenase was down-regulated at 36h, while the corresponding genes were up-regulated (Table 1 and Supplementary Table S1). And only 11 identified proteins showed quantitative agreement with their corresponding genes (a similar fold change in expression levels). However, this variation illustrates the importance of combination of the transcriptomics and proteomics data analysis. The transcriptomics and proteomics data can be used to complement each other in order to avoid analytic variations from each technology, or the detection of a good mRNA-protein correlation can improve the credibility of data analysis.

There are also some limitations in our microarray and 2D results. Due to insufficiency of the Arthrobacter species gene databases, we still have a lot of genes which cannot match any known genes. And the method of 2D gel electrophoresis has many limitations, such as solubility problems and difficulties of detection of low abundance proteins. In our proteomic analysis, 13 out of 67 spots failed in protein identification. Thus further verification and functional analysis are needed to confirm the results of our omics data analysis and to elucidate the molecular mechanism of cAMP production.
Methods

Microorganism, media and culture conditions. The strain used for biosynthesis of cAMP was Arthrobacter sp. CGMCC 3584 that isolated and stored in our laboratory. The seed culture medium contained in grams per liter: glucose 10, peptone 10, beef extract 10, and NaCl 3. The fermentation medium contained in grams per liter: glucose 50, urea 8, K$_2$HPO$_4$ 18, KH$_2$PO$_4$ 5, MgSO$_4$ 0.1, biotin 0.01, hypoxanthine 8, NaF 0.4 and CoCl$_2$ 0.005. The initial pH was adjusted to 7.0 by NaOH and the medium was autoclaved for 20 min at 115°C. The seed cultures were incubated at 30°C and 300 rpm for 18 h. Then the seed medium was transferred into a 5-L fermenter (NBS Bioflo-110) containing 3L of fermentation medium at an inoculum dose of 10% (v/v). The fermentation parameters were controlled by a digital measurement and control system. The cultivation was carried out at 30°C and aeration rate was 8 L/min. The pH was automatically controlled to within 7.0–7.2 with 2.0 M NaOH and 2.0 M HCl. Peanut oil (0.1%, v/v) added as an antifoaming agent. The agitations were set at 150 r/min and 350 r/min to construct low oxygen supply and high oxygen supply conditions, respectively. Tests were carried out in triplicates and the mean values were calculated for fermentation parameters.

Sample collection. Samples (three biological replicates) were respectively collected at 12 h, 24 h, 36 h and 48 h under two different oxygen supply conditions for transcriptional analysis. Equal samples of each of the independent biological replicates were mixed together for DNA microarray analysis. For 2D gel separation, samples (three biological replicates) were respectively collected at 12 h and 36 h under two different oxygen supply conditions. The cells were pelleted by centrifugation at 5000 g for 10 min at 4°C and rinsed twice with ice-cold PBS buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na$_2$HPO$_4$, and 2 mM KH$_2$PO$_4$, pH 7.40). All the cells were frozen immediately using liquid nitrogen and then stored at −80°C.

Genome Sequence. Whole-genome sequencing of Arthrobacter sp. CGMCC 3584 was performed using the Roche 454 Genome Sequencer FLX System (Majorbio, Shanghai, China). After the removal of duplications (by cd-hit-454), the clean data were assembled using GS Assembler. The finished results were analyzed and annotated using Glimmer, GeneMarker, Gismo, epos-blastview, blast2go, tRNAscan-SE, rnammer. The gene function annotation was predicted by using the Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups (COG), and Gene Ontology (GO). Finally, 4,075 coding sequences (CDSs) were predicted and based on this information the following experiments were carried out.

Transcriptome analysis. The Arthrobacter microarrays were customized using Agilent eArray program according to the manufacturer’s recommendations. Each customized microarray (8 × 15 K) contained spots with 4,075 gene-specific 60-mer oligonucleotides representing the 4,075 protein-coding genes in Arthrobacter sp. CGMCC 3584. Total RNA was amplified and labeled by Low Input Quick Amp Labeling Kit, One-Color (Cat#5190-2305, Agilent technologies), following the manufacturer’s instructions. Labeled cRNA were purified by RNeasy mini kit (Cat#74106, QIAGEN). Each Slide was hybridized with 600ng Cy3-labeled cRNA using Gene Expression Hybridization Kit (Cat#5188-5242, Agilent technologies) in Hybridization Oven (Cat#G2545A, Agilent technologies), according to the manufacturer’s instructions. After 17 hours hybridization, slides were washed in staining dishes (Cat#121, Thermo Shandon) with Gene Expression Wash Buffer Kit (Cat#5188-5327, Agilent technologies), followed the manufacturer’s instructions. Slides were scanned by Agilent Microarray Scanner (Cat#G2565CA, Agilent technologies) with default settings, Dye channel: Green, Scan resolution 5µm, PMT 100%, 10%, 16bit. Data were extracted with Feature Extraction software 10.7 (Agilent technologies.). Raw data were normalized by Quantile algorithm, Gene Spring Software 11.0 (Agilent technologies). Genes with fold change bigger than 2 were considered to be significantly differential expression genes responding to different oxygen supply.

qRT-PCR. Five selected genes were further validated by qRT-PCR. Gene-specific primers used for qRT-PCR were listed in Supplementary Table S3. QRT-PCR was carried out by the 7900 HT Sequence Detection System (ABI, USA) using ABI Power SYBR Green PCR Master Mix (ABI, USA) according to the manufacturer’s instructions. The fold change of each transcript in each sample relative to the control sample was measured in triplicates. The 16S rRNA gene was considered as an endogenous reference. And the calculation of differential expression level was based on comparative threshold cycle method

Proteome analysis. The samples were grinded to power with liquid nitrogen and then dissolved in lysis solution at 30°C for 1 hour. The solution was centrifuged by 15000 g for 15 min at room temperature. The clear supernatant was collected and stored at −80°C for isoelectric focusing (IEF). The protein concentration was determined by the bradford method. IEF was carried out on an IPGhor IEF System (GE Healthcare) with 300 μg of protein (450 μl) at 20°C using the 24 cm drystrips (GE Healthcare, pH = 4–7). After equilibration, SDS-PAGE electrophoresis was performed by Etan-DALT-Six system (GE Healthcare) at 15°C. The gel was run for 45 minutes at 100 V then for 6–8 hours at 200 V. The gel was visualized by silver stain as described by Shevchenko et al. The stained gel was then scanned by the Image Scanner (GE Healthcare, USA) at a resolution of 300 dots per inch. All gel images were analyzed using PDquest 8.0 software. A threshold of p ≤ 0.05 and fold change ≥ 2.5 or ≤ 0.4 was used to select differentially protein spots. P-value was performed with Students t test. Selected protein spots were excised from gels and in-gel digestion with trypsin was performed as described previously. Peptide MS and MS/MS were performed on an Ultrasflex TOF/TOF mass spectrometer (Bruker). Both the MS and MS/MS data were integrated by Mascot2.3 (Matrix Science) for protein identification.

Data availability. CDSs description:

1. Gene Expression Omnibus (GEO) accession GPL19020.
2. https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc/GPL19020.
Gene expression data:
1. Gene Expression Omnibus (GEO) accession GSE99546.
2. http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE99546.

KEGG pathway database:
1. Map00010, 00020, 00030, 00230, 00250, 00280, 00290, 00340, 02060.
2. http://www.genome.jp/kegg/pathway.html.

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**Author Contributions**

H.N., Y.C., and H.Y. designed the experiments. H.N., J.W., and W.Z. conducted the experiments. H.N., D.L. performed DNA microarray-based transcriptomic analysis. H.N., J.W. and C.Z. performed proteomic analyses. H.N. and H.Y. wrote the manuscript. All authors read and approved the final manuscript.

**Additional Information**

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**Competing Interests:** The authors declare that they have no competing interests.

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