Title: Oxygen inhibits colibactin production by *Escherichia coli*

Authors : Nadège Bossuet-Greif* [1], Cécile Guyonnet* [1,2], Camille V Chagneau [1,2], Min Tang-Fichaux [1], Marie Penary [1], Priscilla Branchu [1], Eric Oswald [1,2], Jean-Philippe Nougayrede [1]

* *equal contribution*

1 IRSD, INSERM, INRAE, Université de Toulouse, ENVT, Toulouse, France

2 CHU Toulouse, Hôpital Purpan, Service de Bactériologie-Hygiène, Toulouse, France.

Correspondence: jean-philippe.nougayrede@inrae.fr

Abstract :

Commensal and extra-intestinal pathogenic strains of *E. coli* frequently harbor the *pks* genomic island encoding the synthesis of colibactin, a genotoxic metabolite. The gut and infected tissue are low oxygen environments, but it is not known whether oxygen modulates production of the genotoxin. Here, we report on the serendipitous observation that oxygenation of the bacterial culture strongly modulates the autotoxicity of a *E. coli pks clbS* mutant deficient for colibactin self-protection system. We further found that DNA damaging activity by *pks*+ *E. coli* decreased with increasing oxygen concentration in the medium. Similarly, the level of the N-myristoyl-D-Asn metabolite that is released during the final step of colibactin synthesis decreased with increasing oxygen concentration. The activity of the promoter of a gene for the colibactin synthase ClbB, as well as its transcription, were maximal under anoxic conditions, but decreased with increased oxygen concentration. Thus, colibactin synthesis is inhibited by oxygen, suggesting that the *pks* biosynthetic pathway is adapted to the anoxic intestinal lumen and to the hypoxic infected or tumor tissue.

Introduction

Commensal intestinal and pathogenic extraintestinal strains of *E. coli* frequently harbor the *pks* genomic island that allow synthesis of a genotoxin called colibactin (1). The 54 kb *pks* island encodes a complex biosynthesis pathway, with polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS) as well as maturation enzymes, which cooperatively synthesize colibactin. Colibactin is an instable peptide-polyketide metabolite, sensitive to aerobic oxidation and subsequent inactivation (2). Although colibactin is not directly purifiable, its bioactivity has been demonstrated in infected epithelial cells or on DNA exposed to *pks*+ bacteria: colibactin binds covalently to both strands of the DNA helix, resulting in interstrand crosslinks (3–5). These DNA lesions are highly toxic and therefore the *pks* island also encodes self-protection systems (such as the ClbS protein) to defend the producing bacterium from the toxin (6–8). Colibactin is also toxic to other bacteria in the microbial community (9, 10) and to mammalian host cells. Epithelial cells exhibit DNA damage, cell cycle arrest, senescence and death upon exposure to *pks*+ bacteria (11). Colibactin induces apoptotic cell death in lymphocytes and promotes virulence of *pks*+ *E. coli* during sepsis and meningitis (12–14). Colibactin is also produced in the gut by commensal *pks*+ *E. coli* and inflicts DNA damage in intestinal epithelial cells (15–17). This DNA damage can ultimately lead to gene mutagenesis, and colibactin is suspected of promoting colon cancer (17). In mouse models that recapitulate intestinal tumorigenesis, inflammation is essential for cancer promotion by *pks*+ *E. coli*, and inflammation increases the transcription of *pks*-encoded genes (17–19). Thus, these bacteria appear to promote cancer under specific environmental conditions that favor expression of colibactin.

Recent studies have highlighted how environmental conditions regulate colibactin expression in *pks*+ *E. coli*. Indeed, it has been shown that colibactin production is regulated by iron availability (20). Factors originating from the producing bacterium or the host diet also modulate toxin production. Polyamine spermidine from bacterial metabolism or the environment is required for colibactin production (21). Similarly, carbon source impacts *pks* gene expression through regulation of central carbon metabolism (22). Oligosaccharides known to regulate bacterial metabolism increase colibactin expression, but this effect is abrogated by ferrous sulfate supplementation (23). All these studies suggest that colibactin production is constrained by the conditions in the bacterial microniche in the intestinal lumen.

Molecular oxygen (O_2) concentration is a key environmental factor in the gut, playing a central role in metabolic processes of the host and bacteria. The intestinal niche exhibits low oxygen

tension, with concentration ranging from anoxia in the lumen to hypoxia at the epithelial surface (24). This physiological hypoxia can be disrupted during disease, such as chronic inflammation that results in increased epithelial oxygenation favoring aerobic proliferation of *E. coli* (25). This has led to the hypothesis that oxygenation of the intestinal niche is an important factor in the carcinogenic activity of *pks*+ *E. coli* (26). The possibility that oxygen may directly modulate colibactin production by *pks*+ *E. coli* has not yet been studied. In this paper, we report the serendipitous observation that oxygenation of the bacterial culture has a marked impact on the autotoxicity of the genotoxin on the producing bacterium. We further demonstrate that the oxygen concentration in the medium controls colibactin production.

Experimental procedures

Bacterial strains, mutants, plasmids and primers

The bacterial strains, plasmids and primers used in this study are listed in Table 1.

Strains, plasmids, primers	**Genotype, sequence**		**Reference**
E. coli DH10B pBACpks	DH10B strain carrying the *pks*-island on a pBeloBAC11 vector		(27)
DH10B pBACpks *clbS*	*clbS::FRT* mutant		(7)
E. coli SP15	Clinical *E. coli* O18:K1 *pks*+ strain, isolated from spinal fluid of a neonate with meningitis		(16)
pClbS	pASK75 carrying *clbS*		(7)
pCM17	Plasmid containing the luxCDABE operon driven by the *ompC* promoter		(28)
pMT3	pCM17-derived plasmid containing the luxCDABE operon driven by the promoter region of *clbB*		(29)
NG49	Standard range clbB	5'-AAACAACAAGGGGCTGACAC-3'	This study

NG50	Standard range clbB	5'-CAGTCAGCGCCACATGTAAG-3'	This study
NG61	Q-PCR clbB	5'-ACCGAATCAGTATGAAAACCAG-3'	This study
NG62	Q-PCR clbB	5'-GCACACTATCACTCCTCCAAC-3'	This study
NG53	Standard range cysG	5'-CGCCAGCTTTATTATGCCGT-3'	This study
NG54	Standard range cysG	5'-GTAGACCACCACATCTGCCT-3'	This study
NG55	Q-PCR cysG	5'-TGGGCCAGGTAGCGAA-3'	This study
NG56	Q-PCR cysG	5'-GGACCTGCACCAACCA-3'	This study

Bacterial culture conditions

The day before the experiments, the *E. coli* strains were grown overnight at 37°C with 240 RPM shaking in Lennox L broth (LB, Invitrogen) supplemented when required with appropriate antibiotics (Kanamycin 50 µg/ml, streptomycin 50 µg/ml, chloramphenicol 25 µg/ml or carbenicillin 50 µg/ml). Then, the bacteria were pre-cultured in LB or Dulbecco's modified Eagle's medium (DMEM) 25 mM HEPES (Invitrogen) to reach the exponential phase (OD600 ~ 0.4). Culture at various oxygen concentrations were performed in a hypoxic workstation (Whitley H35) regulated at 0.1% to 13% O_2, 5% CO_2, 37°C and 70-90% humidity. To achieve a 0% O_2 anoxic atmosphere, we used a jar with anaerobic atmosphere generation bags (Sigma) manipulated within the hypoxic workstation set at 0.1%. Standard microbiological incubators were used for the 21% O_2 (atmospheric) condition. Culture media were equilibrated for 16-24 h at the required oxygen concentration before use. For DH10B strains, LB cultures were inoculated with $2x10^6$ bacteria/ml in 5 ml and grown overnight (17 h) in fully closed 10 ml tubes (Kima), or in open 50 ml tubes (Falcon), or in 14 ml 17x100 mm tubes with the cap in vented position (Falcon 352059). The *E. coli* strain SP15 was inoculated ($1.5x10^7$ bacteria) in 1 ml DMEM Hepes in Falcon 352059 tubes with the cap in vented position and cultivated 3.5 h with 240 RPM agitation at 37°C within the hypoxystation set at the oxygen concentration required. For CFU enumeration, the cultures were serial-diluted in PBS, plated on LB agar plates and incubated overnight at 37°C in a standard incubator.

Exogenous DNA cross-linking by colibactin-producing *E. coli*

The assay was performed as described before (3), with 100µl of bacterial culture added with 500 ng DNA (pUC19 linearized by BamHI digestion) and 1mM EDTA, then incubated 40 min at the indicated oxygen concentration. The DNA was recovered using a Qiagen PCR purification kit, and analyzed by denaturing gel electrophoresis, stained with GelRed (Biotium) and visualized with a Bio-Rad Chemidoc XRS system. DNA bands were quantified from unsaturated flat-fielded images using ImageJ.

Quantification of N-myristoyl-D-AsnOH (C14-Asn)

Bacterial supernatants (1.5 ml) were prepared by centrifugation at 5000 g, filtration through 0.22 µm PVDF filters (Millipore) and stored at -80°C. Lipids were extracted and analyzed as before (30): briefly, 1 ml of sample was extracted on solid phase 96 wells plates (OASIS HLB, Waters), eluted with methanol, evaporated twice under N_2 then suspended in 10 µl methanol. The quantification of C14-Asn was performed by the MetaToul Lipidomics facility (Inserm UMR1048, Toulouse, France), using an in-house quantification assay by high-performance liquid chromatography/tandem mass spectrometry analysis.

Bioluminescence monitoring of *clbB* promoter activity

The *E. coli* strain SP15 hosting pMT3 or pCM17 (encoding the *Photorhabdus luminescens luxCDABE* operon driven by the *clbB* or *ompC* promoters respectively)(29) was cultivated at the required concentration of oxygen, then 100µl samples were transferred on 96 wells black plates (Greiner) and luminescence was measured with a Tecan Spark plate reader, or visualized with a Bio-Rad Chemidoc XRS system.

***clbB* mRNA quantification**

10^9 bacteria were collected by centrifugation and stored at -80°C. RNA was purified using the RNA Easy plus mini kit (Qiagen), then 2 µg were used to synthesize cDNA with the i-script kit (Bio-Rad). For standard range of *clbB* and *cysG* (housekeeping gene, (31)), PCRs were performed using *E. coli* SP15 genome as a template and the primer pairs NG49/NG50 and NG53/NG54 respectively. Standards were purified and serial diluted from 5.10^9 to 5.10^1 copy/µl. Real time PCR were performed with the iQ SYBR Green supermix (Bio-Rad), with 2 µl of cDNA or standard dilutions, and 187.5 nM of primer pairs NG61/NG62 (*clbB*) or

NG55/NG56 (*cysG*). The copy numbers of *clbB* and *cysG* were calculated from the Ct obtained using the standard curves. The expression of *clbB* gene at different oxygen concentration was calculated by the ratio *clbB* / *cysG* copy numbers and normalized at 100% by the mean at 0,1% oxygen.

Statistical analyses

For quantifications, three independent experiments were performed for each oxygen concentration tested. CFU counts were log-transformed for the analyses. Statistical analyses were performed with GraphPad Prism v9. P values were calculated using one-way ANOVA followed by Tukey post-tests.

Results

Oxygen inhibits colibactin autotoxicity in a *pks*+ *E. coli clbS* mutant

The *pks* biosynthetic pathway encodes self-protective systems, including the ClbS protein that hydrolyzes colibactin and protects the *E. coli* genome DNA from colibactin toxicity (6, 7). We previously observed that a *clbS* mutant in a laboratory strain of *E. coli* producing colibactin exhibits an autotoxicity phenotype, with impaired growth compared to the wild-type strain (7). While studying this *clbS* mutant, we fortuitously observed that its autotoxicity phenotype was sensitive to specific culture conditions, such as tube size, medium volume, and agitation of the culture. In particular, we noticed that the use of culture tubes with tightly or loosely closed caps resulted in autotoxicity or normal growth, respectively. Based on this observation, we found that the *clbS* mutant showed a significant ~10-fold decrease in CFU count compared with wild-type or complemented mutant strains when grown in small closed tubes, but not in large open tubes (Fig 1A). We thought that differences in culture oxygenation were a likely explanation for this observation.

To confirm a role of oxygen in the expression of the *clbS* mutant autotoxicity phenotype, we tested cultures under atmospheric or hypoxic conditions. Culture tubes with vented caps were incubated with agitation in a standard atmosphere incubator (21% O_2) or in a hypoxic chamber with a 0.1% O_2 atmosphere. In the hypoxic chamber, the *clbS* mutant showed a marked decrease of approximately 100-fold in CFU numbers compared with the wild type or the complemented

mutant (Fig 1B). In contrast, the *clbS* culture in the 21% O_2 incubator did not show such a decrease in growth.

Oxygen inhibits DNA crosslinking by *E. coli* producing colibactin

The reduced autotoxicity phenotype in the *clbS* mutant exposed to high oxygen concentration suggests that colibactin production, and/or its genotoxic activity, was inhibited. We therefore examined the effect of various oxygen concentrations on colibactin production and activity. Mature colibactin cannot be purified, but its genotoxicity can be detected by its DNA cross-linking activity on exogenous DNA exposed to *pks*+ *E. coli* bacteria (3). We used the human clinical *pks*+ *E. coli* strain SP15, which has been previously shown to produce colibactin in the mouse intestinal lumen (16, 26) and to alter the gut microbiota through the production of colibactin (32). DNA cross-linking activity of *E. coli* strain SP15 on linear DNA was tested at different oxygen concentrations (0-21%). DNA cross-linking by colibactin was readily detected at low (<1%) oxygen concentrations but decreased significantly at higher (4 and 13%) concentrations, becoming undetectable at 21% O_2 (Figure 2AB). The growth of *E. coli* strain SP15 was similar regardless of oxygen concentration (Figure 2C), indicating that the modulation of its cross-linking activity was not explained by the number of bacteria. Thus, oxygen inhibits the activity and/or production of colibactin.

Oxygen inhibits expression and production of colibactin

To examine whether oxygen modulates colibactin production, we quantified the metabolite N-myristoyl-D-AsnOH (C14-Asn), a stable byproduct released during the final step of colibactin synthesis (8). *E. coli* strain SP15 was grown as before with different oxygen concentrations, lipids were extracted from the culture supernatants and analyzed by quantitative chromatography coupled to mass spectrometry. C14-Asn production by SP15 was highest at low oxygen concentrations (<1% O_2) and decreased at higher concentrations, with only background levels at 13 and 21% O_2 (Figure 3). This result indicated that synthesis of colibactin is the highest at low oxygen concentration but reduced with increasing oxygen concentration.

To confirm that oxygen inhibits colibactin synthesis, we measured the activity of the promoter of *clbB*, which encodes one of the key NRPS-PKSs in the synthesis pathway (29). *E. coli* strain SP15 harboring a luciferase reporter system under the control of either the *ompC* or *clbB* promoters was grown with different oxygen concentrations, and then luminescence was

quantified (Figure 4). The *ompC* constitutive promoter showed no significant change in its activity at all oxygen concentrations. In contrast, the *clbB* promoter showed its highest activity in anoxic cultures, and decreasing activity with increasing oxygen concentration (Figure 4). We also measured the expression of the *clbB* gene and a control reference gene (*cysG*) by quantitative PCR. We found that *clbB* mRNA expression was highest in anoxic cultures and decreased with increasing oxygen concentration (Figure 5). Together with the observation that DNA cross-linking activity and colibactin autotoxicity are inhibited by oxygen, these results demonstrated that oxygen constrains colibactin production.

Discussion

One of the most notable characteristics of colibactin is its instability, which complicates its purification and probably explains the need for close proximity between *pks*+ bacteria and target cells to inflict DNA damage (2, 27). Indeed, synthetic and chemical studies shown that colibactin is susceptible to aerobic oxidation and subsequent inactivation. Here, we demonstrated that oxygen reduces the production of colibactin by decreasing the expression of NRPS-PKS *clbB* gene at the promoter and transcript level. The latter result is consistent with a recent report of increased transcription of a *clbB-lacZ* reporter under oxygen-limited conditions (9). Together, these findings show that oxygen not only degrades colibactin, but also inhibits its synthesis by *pks*+ *E. coli*.

Inhibition of colibactin production by oxygen is consistent with the observation that fitness and virulence systems of enterobacteria are typically regulated by oxygen. Indeed, commensal as well as pathogenic enterobacteria encounter oxygen levels that are generally hypoxic, with steep gradients and dynamic oxygenation cycles. Natural oxygenation conditions are in sharp contrast to the constant, high oxygen concentration used in standard "normoxic" (21% O_2) *in vitro* experiments. In contrast, the intestine exhibit longitudinal and cross-sectional gradients of oxygen concentration, decreasing from the small to the large intestine, and increasing from the anoxic (<0.1% O_2) lumen to the oxygenated (~1% O_2) epithelium surface (33). Intestinal oxygenation further fluctuates between rest and ingestion of nutrients, when absorptive hyperemia occurs. In the host epithelial tissues and fluids (such as urine), pathogenic enterobacteria encounter “physioxic” oxygen levels (2% to 9%, averaging ~4%) (34). Pathogenic enterobacteria such as *Shigella*, enterotoxigenic *E. coli* and uropathogenic *E. coli* tightly regulate the expression of their virulence factors in relation to oxygen concentration (35–

37). For *pks*+ *E. coli*, the inhibition of colibactin production in the presence of oxygen could be viewed as a selected advantage. Indeed, the metabolic cost of synthesizing a complex compound such as colibactin using eight huge NRPS and PKS enzymes is very high, implying that the selective pressure for their expression and maintenance must be strong (38). As colibactin is susceptible to aerobic oxidation, an energy saving strategy that inhibits its synthesis under environmental oxygen conditions leading to its inactivation may have been selected. Alternatively, the adaptation of colibactin bioactivity and production to a low-oxygen environment could be due to the role of colibactin in the competition with the polymicrobial community in the anoxic intestinal lumen. The adaptation of colibactin bioactivity and production to a low-oxygen environment could be due to the role of colibactin in the competition with the polymicrobial community in the anoxic intestinal lumen. Indeed, recent findings highlighted a direct role for colibactin in killing or inhibiting enteric bacteria (such as the anaerobe *Bacteroides fragilis*), in the induction of prophages in polymicrobial enteric anerobic cultures, and ultimately in shaping the gut microbiota (9, 10, 32). Our finding that an anoxic environment is most favorable for colibactin production is thus consistent with the recently proposed role of colibactin in interbacterial competition in gut lumen.

Our results are also relevant to the question of where and when colibactin inflicts DNA damage in host epithelial cells. It has been repeatedly reported that DNA damage was observed in enterocytes of rats and mice colonized or force-fed with different strains of *E. coli pks*+ (NC101, 192PP, M1/5, SP15, Nissle 1917) (13, 15, 16, 18, 39). Keeping in mind that colibactin production is also regulated by numerous environmental cues such as iron, polyamines, and carbon sources (20–22), the physiological hypoxia at the surface of the epithelium could constitute a favorable environment for colibactin production and activity. Other low oxygen intestinal compartments could favor colibactin production, such as the stem cells that reside in a hypoxic niche (as low as 1% O_2) at the bottom of the crypt (40). Signs of long-term persistent chromosomal instability have been reported in animals colonized with *pks*+ *E. coli* (39) and the mutational signature of colibactin has been found in intestinal crypts in healthy human subjects (41), suggesting that *pks*+ *E. coli* could leave their imprint in stem cells. *E. coli pks*+ have not yet been observed in the stem niche, but were shown to form biofilms together with the anaerobe *B. fragilis* on the colonic epithelium of colorectal cancer patients (42). It is well known that tumor tissue is hypoxic compared to healthy tissue (43), and thus could present another favorable environment for colibactin production. It is also interesting to note that inflammation appears to be a key factor in promoting colorectal tumorigenesis by *E. coli pks*+ in mouse models (17, 18). Severe inflammation can lead to local oxygen depletion, resulting from tissue

necrosis and neutrophil respiratory burst (44). Such hypoxic areas can also be generated during infectious processes, when the host responds by coagulation to prevent bacterial dissemination, thus generating a rapid drop in local oxygen tension (45). Hypoxia at the level of inflamed and infected tissues could therefore present environments favorable for colibactin production. Further study of the localization of *pks*+ bacteria in relation to host cell DNA damage and tissue inflammation will be necessary to better understand the impact of colibactin on infection and cancer.

In conclusion, we provide evidence that oxygen decreases colibactin synthesis by *E. coli*, prompting the hypothesis that colibactin production and genotoxic activity are adapted to the bacterial warfare in the anoxic gut and also to the hypoxic infected, inflamed and tumor tissues.

Funding information

This work was supported by the French Agence Nationale de la Recherche under grants ANR-17-CE35-0010, ANR-18-CE14-0039, ANR-19-CE34-0014. The hypoxystation used in this work was sponsored by a grant from INRAE (to JPN) and the European Regional Development Fund (to Nathalie Vergnolle, IRSD). CG was a recipient of a scholarship “Année de recherche”, inter-région Sud-Ouest, filière pharmacie, 2019.

Disclosure statement

The authors report there are no competing interests to declare.

References

1. Nougayrède J-P, Homburg S, Taieb F, Boury M, Brzuszkiewicz E, Gottschalk G, Buchrieser C, Hacker J, Dobrindt U, Oswald E. 2006. Escherichia coli induces DNA double-strand breaks in eukaryotic cells. 5788. Science 313:848–51.

2. Wernke KM, Xue M, Tirla A, Kim CS, Crawford JM, Herzon SB. 2020. Structure and bioactivity of colibactin. Bioorg Med Chem Lett 30:127280.

3. Bossuet-Greif N, Vignard J, Taieb F, Mirey G, Dubois D, Petit C, Oswald E, Nougayrède J-P. 2018. The Colibactin Genotoxin Generates DNA Interstrand Cross-Links in Infected Cells. 2. mBio 9:e02393-17.

4. Xue M, Kim CS, Healy AR, Wernke KM, Wang Z, Frischling MC, Shine EE, Wang W, Herzon SB, Crawford JM. 2019. Structure elucidation of colibactin and its DNA cross-links. Science 365.

5. Wilson MR, Jiang Y, Villalta PW, Stornetta A, Boudreau PD, Carrá A, Brennan CA, Chun E, Ngo L, Samson LD, Engelward BP, Garrett WS, Balbo S, Balskus EP. 2019. The human gut bacterial genotoxin colibactin alkylates DNA. Science 363.

6. Tripathi P, Shine EE, Healy AR, Kim CS, Herzon SB, Bruner SD, Crawford JM. 2017. ClbS Is a Cyclopropane Hydrolase That Confers Colibactin Resistance. 49. JOURNAL OF THE AMERICAN CHEMICAL SOCIETY 139:17719–17722.

7. Bossuet-Greif N, Dubois D, Petit C, Tronnet S, Martin P, Bonnet R, Oswald E, Nougayrède J-P. 2016. Escherichia coli ClbS is a colibactin resistance protein. 5. Mol Microbiol 99:897–908.

8. Brotherton CA, Balskus EP. 2013. A prodrug resistance mechanism is involved in colibactin biosynthesis and cytotoxicity. 9. J Am Chem Soc 135:3359–3362.

9. Chen J, Byun H, Liu R, Jung I-J, Pu Q, Zhu CY, Tanchoco E, Alavi S, Degnan PH, Ma AT, Roggiani M, Beld J, Goulian M, Hsiao A, Zhu J. 2022. A commensal-encoded genotoxin drives restriction of Vibrio cholerae colonization and host gut microbiome remodeling. Proc Natl Acad Sci U S A 119:e2121180119.

10. Silpe JE, Wong JWH, Owen SV, Baym M, Balskus EP. 2022. The bacterial toxin colibactin triggers prophage induction. Nature 603:315–320.

11. Faïs T, Delmas J, Barnich N, Bonnet R, Dalmasso G. 2018. Colibactin: More Than a New Bacterial Toxin. Toxins (Basel) 10:E151.

12. Marcq I, Martin P, Payros D, Cuevas-Ramos G, Boury M, Watrin C, Nougayrède J-P, Olier M, Oswald E. 2014. The genotoxin colibactin exacerbates lymphopenia and decreases survival rate in mice infected with septicemic Escherichia coli. 2. J Infect Dis 210:285–294.

13. McCarthy AJ, Martin P, Cloup E, Stabler RA, Oswald E, Taylor PW. 2015. The Genotoxin Colibactin Is a Determinant of Virulence in Escherichia coli K1 Experimental Neonatal Systemic Infection. 9. Infect Immun 83:3704–3711.

14. Wang P, Zhang J, Chen Y, Zhong H, Wang H, Li J, Zhu G, Xia P, Cui L, Li J, Dong J, Gao Q, Meng X. 2021. Colibactin in avian pathogenic Escherichia coli contributes to the development of meningitis in a mouse model. Virulence 12:2382–2399.

15. Nougayrède J-P, Chagneau CV, Motta J-P, Bossuet-Greif N, Belloy M, Taieb F, Gratadoux J-J, Thomas M, Langella P, Oswald E. 2021. A Toxic Friend: Genotoxic and Mutagenic Activity of the Probiotic Strain Escherichia coli Nissle 1917. mSphere 6:e0062421.

16. Cuevas-Ramos G, Petit CR, Marcq I, Boury M, Oswald E, Nougayrède J-P. 2010. Escherichia coli induces DNA damage in vivo and triggers genomic instability in mammalian cells. 25. Proc Natl Acad Sci USA 107:11537–11542.

17. Dougherty MW, Jobin C. 2021. Shining a Light on Colibactin Biology. Toxins (Basel) 13:346.

18. Arthur JC, Perez-Chanona E, Mühlbauer M, Tomkovich S, Uronis JM, Fan T-J, Campbell BJ, Abujamel T, Dogan B, Rogers AB, Rhodes JM, Stintzi A, Simpson KW, Hansen JJ, Keku TO, Fodor AA, Jobin C. 2012. Intestinal inflammation targets cancer-inducing activity of the microbiota. 6103. Science 338:120–123.

19. Arthur JC, Gharaibeh RZ, Mühlbauer M, Perez-Chanona E, Uronis JM, McCafferty J, Fodor AA, Jobin C. 2014. Microbial genomic analysis reveals the essential role of inflammation in bacteria-induced colorectal cancer. Nat Commun 5:4724.

20. Tronnet S, Garcie C, Rehm N, Dobrindt U, Oswald E, Martin P. 2016. Iron Homeostasis Regulates the Genotoxicity of Escherichia coli That Produces Colibactin. Infect Immun 84:3358–3368.

21. Chagneau CV, Garcie C, Bossuet-Greif N, Tronnet S, Brachmann AO, Piel J, Nougayrède J-P, Martin P, Oswald E. 2019. The Polyamine Spermidine Modulates the Production of the Bacterial Genotoxin Colibactin. mSphere 4:e00414-19.

22. Rehm N, Wallenstein A, Keizers M, Homburg S, Magistro G, Chagneau CV, Klimek H, Revelles O, Helloin E, Putze J, Nougayrède J-P, Schubert S, Oswald E, Dobrindt U. 2022. Two Polyketides Intertwined in Complex Regulation: Posttranscriptional CsrA-Mediated Control of Colibactin and Yersiniabactin Synthesis in Escherichia coli. mBio e0381421.

23. Oliero M, Calvé A, Fragoso G, Cuisiniere T, Hajjar R, Dobrindt U, Santos MM. 2021. Oligosaccharides increase the genotoxic effect of colibactin produced by pks+ Escherichia coli strains. BMC Cancer 21:172.

24. Zheng L, Kelly CJ, Colgan SP. 2015. Physiologic hypoxia and oxygen homeostasis in the healthy intestine. A Review in the Theme: Cellular Responses to Hypoxia. Am J Physiol Cell Physiol 309:C350-360.

25. Byndloss MX, Bäumler AJ. 2018. The germ-organ theory of non-communicable diseases. Nat Rev Microbiol 16:103–110.

26. Cevallos SA, Lee J-Y, Tiffany CR, Byndloss AJ, Johnston L, Byndloss MX, Bäumler AJ. 2019. Increased Epithelial Oxygenation Links Colitis to an Expansion of Tumorigenic Bacteria. mBio 10:e02244-19.

27. Nougayrède J-P, Homburg S, Taieb F, Boury M, Brzuszkiewicz E, Gottschalk G, Buchrieser C, Hacker J, Dobrindt U, Oswald E. 2006. Escherichia coli induces DNA double-strand breaks in eukaryotic cells. Science 313:848–851.

28. Rhee K-J, Cheng H, Harris A, Morin C, Kaper JB, Hecht G. 2011. Determination of spatial and temporal colonization of enteropathogenic E. coli and enterohemorrhagic E. coli in mice using bioluminescent in vivo imaging. Gut Microbes 2:34–41.

29. Tang-Fichaux M, Chagneau CV, Bossuet-Greif N, Nougayrède J-P, Oswald É, Branchu P. 2020. The Polyphosphate Kinase of Escherichia coli Is Required for Full Production of the Genotoxin Colibactin. mSphere 5:e01195-20.

30. Pérez-Berezo T, Pujo J, Martin P, Le Faouder P, Galano J-M, Guy A, Knauf C, Tabet JC, Tronnet S, Barreau F, Heuillet M, Dietrich G, Bertrand-Michel J, Durand T, Oswald E,

Cenac N. 2017. Identification of an analgesic lipopeptide produced by the probiotic Escherichia coli strain Nissle 1917. Nat Commun 8:1314.

31. Zhou K, Zhou L, Lim Q 'En, Zou R, Stephanopoulos G, Too H-P. 2011. Novel reference genes for quantifying transcriptional responses of Escherichia coli to protein overexpression by quantitative PCR. BMC Mol Biol 12:18.

32. Tronnet S, Floch P, Lucarelli L, Gaillard D, Martin P, Serino M, Oswald E. 2020. The Genotoxin Colibactin Shapes Gut Microbiota in Mice. mSphere 5:e00589-20.

33. Albenberg L, Esipova TV, Judge CP, Bittinger K, Chen J, Laughlin A, Grunberg S, Baldassano RN, Lewis JD, Li H, Thom SR, Bushman FD, Vinogradov SA, Wu GD. 2014. Correlation between intraluminal oxygen gradient and radial partitioning of intestinal microbiota. Gastroenterology 147:1055-1063.e8.

34. Brahimi-Horn MC, Pouysségur J. 2007. Oxygen, a source of life and stress. FEBS Lett 581:3582–3591.

35. Crofts AA, Giovanetti SM, Rubin EJ, Poly FM, Gutiérrez RL, Talaat KR, Porter CK, Riddle MS, DeNearing B, Brubaker J, Maciel M, Alcala AN, Chakraborty S, Prouty MG, Savarino SJ, Davies BW, Trent MS. 2018. Enterotoxigenic E. coli virulence gene regulation in human infections. Proc Natl Acad Sci USA 115:E8968–E8976.

36. Marteyn B, West NP, Browning DF, Cole JA, Shaw JG, Palm F, Mounier J, Prévost M-C, Sansonetti P, Tang CM. 2010. Modulation of Shigella virulence in response to available oxygen in vivo. Nature 465:355–358.

37. Eberly AR, Floyd KA, Beebout CJ, Colling SJ, Fitzgerald MJ, Stratton CW, Schmitz JE, Hadjifrangiskou M. 2017. Biofilm Formation by Uropathogenic Escherichia coli Is Favored under Oxygen Conditions That Mimic the Bladder Environment. Int J Mol Sci 18:E2077.

38. Fischbach MA, Walsh CT, Clardy J. 2008. The evolution of gene collectives: How natural selection drives chemical innovation. Proceedings of the National Academy of Sciences 105:4601–4608.

39. Payros D, Secher T, Boury M, Brehin C, Ménard S, Salvador-Cartier C, Cuevas-Ramos G, Watrin C, Marcq I, Nougayrède J-P, Dubois D, Bedu A, Garnier F, Clermont O, Denamur E, Plaisancié P, Theodorou V, Fioramonti J, Olier M, Oswald E. 2014. Maternally acquired genotoxic Escherichia coli alters offspring's intestinal homeostasis. 3. Gut Microbes 5:313–325.

40. Mohyeldin A, Garzón-Muvdi T, Quiñones-Hinojosa A. 2010. Oxygen in stem cell biology: a critical component of the stem cell niche. Cell Stem Cell 7:150–161.

41. Lee-Six H, Olafsson S, Ellis P, Osborne RJ, Sanders MA, Moore L, Georgakopoulos N, Torrente F, Noorani A, Goddard M, Robinson P, Coorens THH, O'Neill L, Alder C, Wang J, Fitzgerald RC, Zilbauer M, Coleman N, Saeb-Parsy K, Martincorena I, Campbell PJ, Stratton MR. 2019. The landscape of somatic mutation in normal colorectal epithelial cells. Nature 574:532–537.

42. Dejea CM, Fathi P, Craig JM, Boleij A, Taddese R, Geis AL, Wu X, DeStefano Shields CE, Hechenbleikner EM, Huso DL, Anders RA, Giardiello FM, Wick EC, Wang H, Wu S, Pardoll DM, Housseau F, Sears CL. 2018. Patients with familial adenomatous polyposis harbor colonic biofilms containing tumorigenic bacteria. 6375. Science 359:592–597.

43. McKeown SR. 2014. Defining normoxia, physoxia and hypoxia in tumours-implications for treatment response. Br J Radiol 87:20130676.

44. Campbell EL, Bruyninckx WJ, Kelly CJ, Glover LE, McNamee EN, Bowers BE, Bayless AJ, Scully M, Saeedi BJ, Golden-Mason L, Ehrentraut SF, Curtis VF, Burgess A, Garvey JF, Sorensen A, Nemenoff R, Jedlicka P, Taylor CT, Kominsky DJ, Colgan SP. 2014. Transmigrating neutrophils shape the mucosal microenvironment through localized oxygen depletion to influence resolution of inflammation. Immunity 40:66–77.

45. Melican K, Boekel J, Månsson LE, Sandoval RM, Tanner GA, Källskog Ö, Palm F, Molitoris BA, Richter-Dahlfors A. 2008. Bacterial infection-mediated mucosal signalling induces local renal ischaemia as a defence against sepsis. Cellular Microbiology 10:1987–1998.

Figures

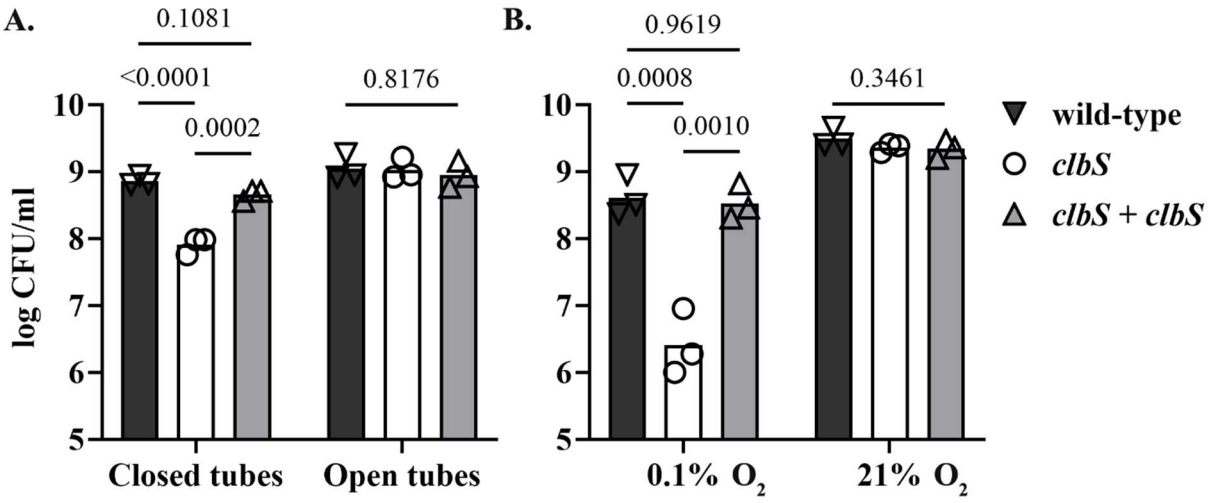

Figure 1: Culture oxygenation modulates the autotoxicity phenotype of a *clbS* mutant in a *pks*+ *E. coli*.

A. *E. coli* strain DH10B pBAC*pks*, the isogenic *clbS* mutant deficient for colibactin self-resistance protein, and the trans-complemented *clbS* mutant (*clbS* + *clbS*), were grown (5 ml LB) with shaking in a standard atmospheric incubator, in 10 ml tubes that were tightly closed, or in 50 ml tubes with the cap opened. **B.** The bacteria were cultured with shaking in tubes with cap in vented position, within a hypoxic chamber at 0.1% O_2, or in a standard atmosphere incubator (21% O_2).

After 16 h culture, bacterial growth was determined by plating and counting colony forming units (CFU). The mean and individual results of three independent experiments are shown, with the p values of an ANOVA and Tukey's multiple comparison test.

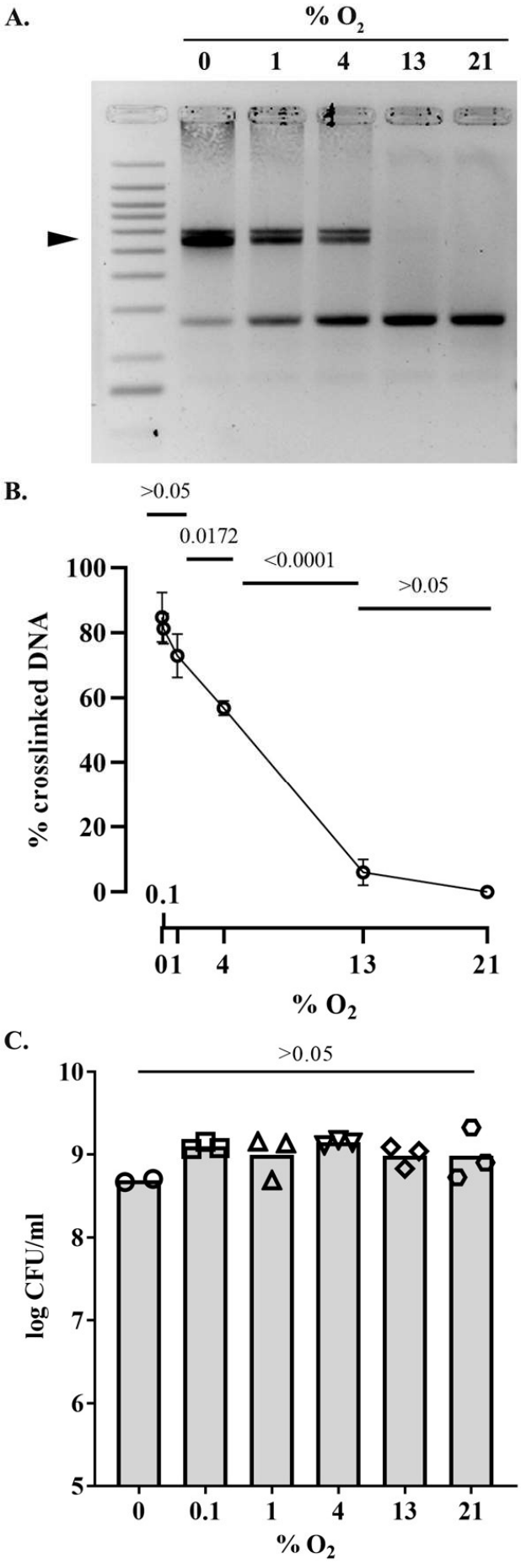

Figure 2: Effect of oxygen concentration on the DNA crosslinking activity of a *pks*+ *E. coli.*

A. Linearized plasmid DNA was exposed 40 min to the *pks*+ *E. coli* strain SP15 grown 3.5 hours with shaking in vented cap tubes within an incubator regulated at the given percentage of oxygen, and then analyzed by denaturing gel electrophoresis. The DNA cross-linked by colibactin with apparent doubling in size compared to the intact denatured DNA is shown with an arrow.

B. The percentage of DNA signal in the upper, crosslinked DNA relative to the total DNA signal in the lane was determined by image analysis. The mean and standard error of three independent experiments are shown, with the p values of an ANOVA and Tukey's multiple comparison test.

C. In the same experiments, the bacterial growth following 3.5 hours culture at given percentages of oxygen was examined by plating and counting colony forming units (CFU).

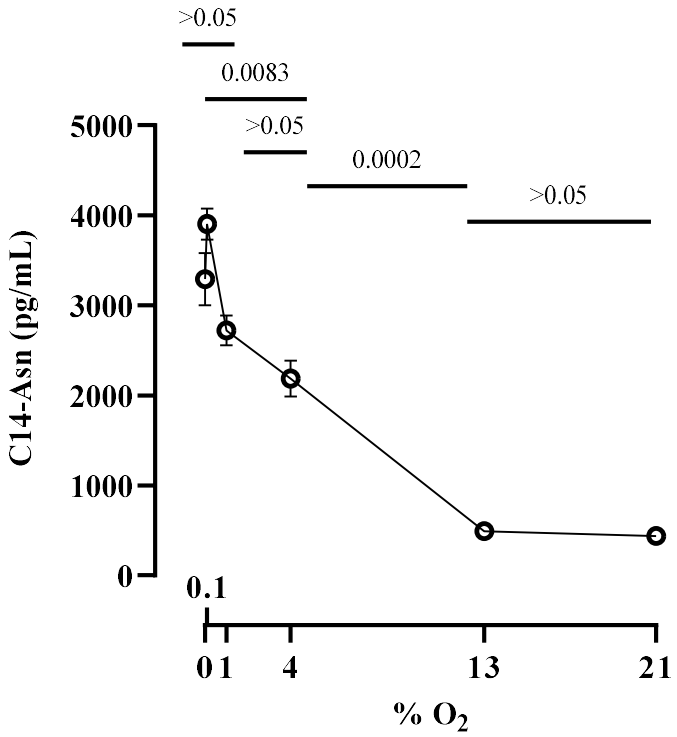

Figure 3 : Effect of oxygen concentration on the production of colibactin cleavage product C14-Asn.

The *pks*+ *E. coli* strain SP15 was grown 3.5 hours with shaking in vented tubes in an incubator regulated at various percentage of oxygen, then the culture supernatants were collected, the lipids were extracted and colibactin cleavage product C14-Asn was quantified by liquid chromatography coupled to mass spectrometry. The mean and standard error of three independent cultures are shown, with the p values of an ANOVA and Tukey's multiple comparison test. The error bars of the biological triplicate samples at $\%O_2$>13% are too small to appear on the graph.

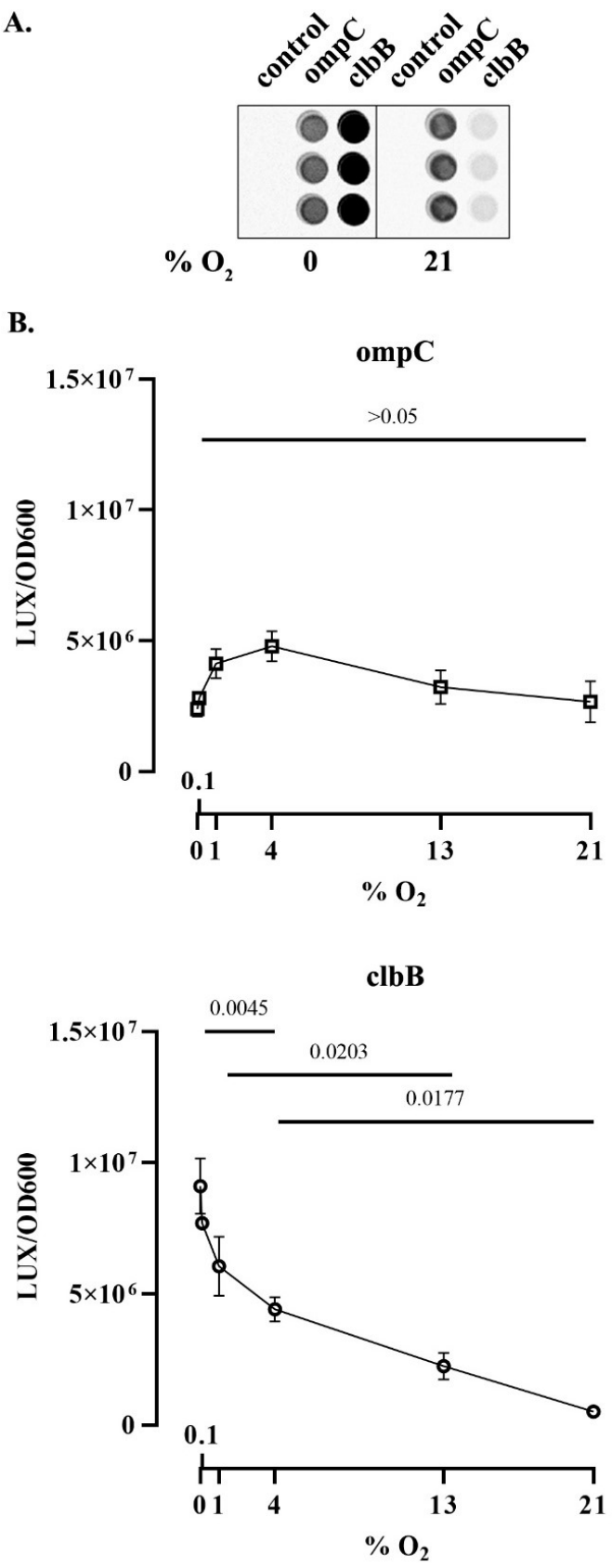

Figure 4 : Effect of oxygen concentration on *clbB* promoter activity.

A. Luminescence of *pks*+ *E. coli* strain SP15 carrying a luciferase reporter under control of *ompC* or *clbB* promoters, or without promoter as a control. Triplicate bacterial culture (100 µl) following 3.5h growth at 0% or 21% of oxygen were placed in a 96-well microplate and photographed with a CCD camera in a dark imaging station. Atmospheric oxygen present during the image acquisition was sufficient to provide oxygen to the luciferase.

B. The luminescence (normalized to the bacterial optical density at 600 nm) was measured in a plate reader following 3.5 hours culture at specified percentages of oxygen. The mean and standard error of three independent cultures are shown, with the p values of an ANOVA and Tukey's multiple comparison test.

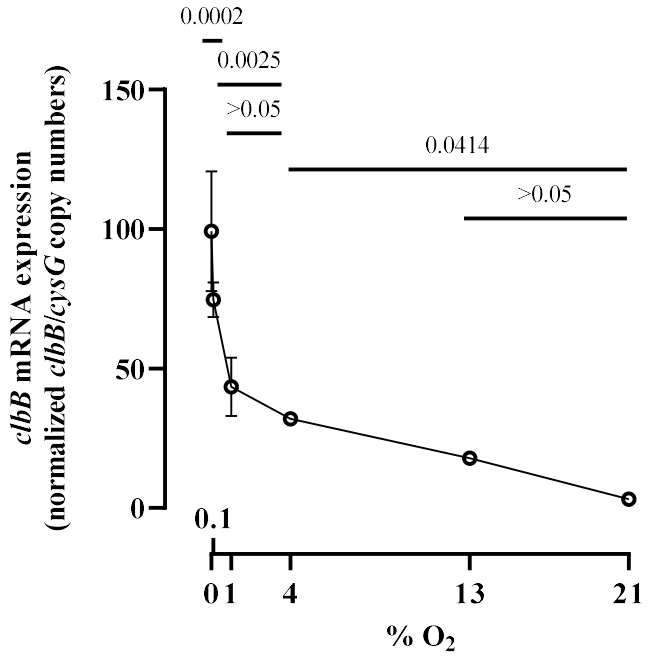

Figure 5 : Effect of oxygen concentration on *clbB* gene expression.
The mRNA levels of *clbB* gene were determined in *E. coli* SP15 grown 3.5 hours at specified percentages of oxygen. The DNA copy number of the *clbB* mRNA relative to that of the *cysG* housekeeping gene was normalized to the mean maximum level. The mean and standard error of three independent cultures are shown, with the p values of an ANOVA and Tukey's multiple comparison test. The error bars of the biological triplicate samples at $\%O_2$>4% are too small to appear on the graph.