Title: Oxygen concentration modulates colibactin production

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Abstract :

Up to 25% of the *E. coli* strains isolated from the feces of healthy humans harbor the *pks* genomic island encoding the synthesis of colibactin, a genotoxic metabolite. Evidence is accumulating for an etiologic role of colibactin in colorectal cancer. Little is known about the conditions of expression of colibactin in the gut. The intestine is characterized by a unique oxygenation profile, with a steep gradient between the physiological hypoxic epithelial surface and the anaerobic lumen, which favors the dominance of obligate anaerobes. Here, we report that colibactin production is maximal under anoxic conditions and decreases with increased oxygen concentration. Deletion of the *arcA* gene reduces the genotoxicity of *pks*+ *E. coli* in infected epithelial cells, indicating that the aerobic respiration control (Arc) system positively regulates colibactin production. 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.

Keywords

Escherichia coli, colibactin, DNA damage, oxygen, hypoxia

Introduction

E. coli strains isolated from the feces of healthy subjects or from extra-intestinal infections, frequently harbor the *pks* genomic island that allow synthesis of a genotoxin called colibactin (1). This 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). Because these DNA lesions are highly toxic, 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 the bacteria in the microbial community (9, 10) and to host mammalian 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 *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 genotoxin production. The 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). Together, these studies suggest that colibactin production is constrained by the conditions in the bacterial microniche in the intestinal lumen.

61 The concentration of molecular oxygen (O_2, hereafter referred to as oxygen) is a key
62 environmental factor in the gut, playing a central role in metabolic processes of both the host
63 and intestinal bacteria. The intestinal niche exhibits low oxygen tension, with a steep gradient
64 from anoxia in the lumen to physiological hypoxia at the epithelial surface (24). Oxygen serves
65 as an environmental cue for *E. coli*, with the Anoxic Redox Control (or Aerobic Respiration
66 Control; Arc) system detecting oxygen deprivation to regulate the expression of anaerobic
67 adaptation genes (25). The physiological hypoxia in the gut can be disrupted during disease,
68 such as at sites of mucosal inflammatory lesions, or in tumors, which can be deeply hypoxic
69 (26, 27) . On the other hand, chronic inflammation results in increased epithelial oxygenation
70 favoring aerobic proliferation of *E. coli* (28, 29). This has led to the hypothesis that oxygenation
71 of the intestinal niche is an important factor in the carcinogenic activity of *pks+ E. coli* (29).
72 The possibility that oxygen may directly modulate colibactin production by *pks+ E. coli* has
73 not yet been studied. In this paper, we report the serendipitous observation that oxygenation of
74 the bacterial culture has a marked impact on the autotoxicity of the genotoxin on the producing
75 bacterium. We further demonstrate that oxygen inhibits colibactin production and genotoxicity
76 in infected epithelial cells, and that the Arc system is a positive regulator of colibactin
77 production.

78

79

80 **Results**

81

82 **Oxygen inhibits colibactin autotoxicity in a colibactin-producing *E. coli clbS* mutant**

83

84 The colibactin biosynthetic pathway encodes self-protective systems, including the ClbS
85 protein that hydrolyzes colibactin and protects the *E. coli* genome DNA from colibactin toxicity
86 (6, 7). We previously observed that a laboratory K12 strain of *E. coli* transformed with a BAC
87 harboring the *pks* island with a deleted *clbS* gene exhibits an autotoxicity phenotype, with
88 impaired growth compared to the control strain harboring the intact *pks* island (7). While
89 studying this *clbS* mutant, we fortuitously observed that its autotoxicity phenotype was sensitive
90 to specific culture conditions. In particular, we noticed that the use of culture tubes with tightly
91 or loosely closed caps resulted in autotoxicity or normal growth, respectively. Based on this
92 observation, we found that the *clbS* mutant showed a significant ~10-fold decrease in CFU
93 count compared with wild-type or complemented mutant strains when grown in small closed

94 tubes, but not in large open tubes (Figure 1A). We thought that differences in culture
95 oxygenation were a likely explanation for this observation. To confirm a role of oxygen in the
96 expression of the *clbS* mutant autotoxicity phenotype, we tested cultures under atmospheric or
97 hypoxic conditions. Culture tubes with vented caps were incubated with agitation in a standard
98 atmosphere incubator (21% O_2) or in a dedicated hypoxic chamber with a 0.1% O_2 atmosphere.
99 In the hypoxic chamber, the *clbS* mutant showed a marked decrease of approximately 100-fold
100 in CFU numbers compared with the wild type or the complemented mutant (Figure 1B). In
101 contrast, the *clbS* culture in the 21% O_2 incubator did not show such a decrease in growth.
102
103 **Oxygen inhibits DNA-crosslinking by *pks+ E. coli***
104 The reduced autotoxicity phenotype in the *clbS* mutant exposed to high oxygen concentration
105 suggested that colibactin production, and/or its genotoxic activity, was inhibited. We therefore
106 examined the effect of various oxygen concentrations on colibactin production and activity.
107 Mature colibactin cannot be purified, but its genotoxicity can be detected by its DNA cross-
108 linking activity on exogenous DNA exposed to *pks+ E. coli* bacteria (3). We used the human
109 clinical *pks+ E. coli* strain SP15, which has been previously shown to produce colibactin in the
110 mouse intestinal lumen (16, 29) and to alter the gut microbiota through the production of
111 colibactin (30). DNA cross-linking activity of *E. coli* strain SP15 on linear DNA was tested at
112 different oxygen concentrations (0-21%). DNA cross-linking by colibactin was readily detected
113 at low (<1%) oxygen concentrations but decreased markedly at higher (4 and 13%)
114 concentrations, becoming undetectable at 21% O_2 (Figure 2AB). The growth of *E. coli* strain
115 SP15 was similar regardless of oxygen concentration (Figure 2C), indicating that the
116 modulation of its cross-linking activity was not explained by the number of bacteria. Thus,
117 oxygen inhibits the activity and/or production of colibactin.
118
119 **Oxygen inhibits the expression and production of colibactin by *pks+ E. coli***
120 To examine whether oxygen modulates colibactin production, we quantified the metabolite N-
121 myristoyl-D-AsnOH (C14-Asn), a stable byproduct released during the final step of colibactin
122 synthesis (8). *E. coli* strain SP15 was grown as before with different oxygen concentrations,
123 lipids were extracted from the culture supernatants and analyzed by quantitative
124 chromatography coupled to mass spectrometry. C14-Asn production by SP15 was highest at
125 low oxygen concentrations (<1% O_2) and decreased at higher concentrations, with only
126 background levels at 13 and 21% O_2 (Figure 3). Thus, synthesis of colibactin was the highest
127 at low oxygen concentration but diminished with increasing oxygen concentration.

128 To confirm that oxygen inhibits colibactin synthesis, we measured the activity of the
129 promoter of *clbB*, which encodes one of the key NRPS-PKSs in the synthesis pathway (31). *E.*
130 *coli* strain SP15 harboring a luciferase reporter system under the control of either a known
131 constitutive promoter or the *clbB* promoter was grown with different oxygen concentrations,
132 and then luminescence was quantified (Figure 4). The constitutive promoter showed no
133 significant change in activity at all oxygen concentrations, whereas the *clbB* promoter showed
134 its highest activity in anoxic cultures, and decreasing activity with increasing oxygen
135 concentration (Figure 4). This result is consistent with a recent report of increased transcription
136 of a *clbB-lacZ* reporter under oxygen-limited conditions (9). To confirm this finding, we
137 measured by quantitative PCR the expression of the *clbB* gene and that of a control reference
138 gene. We found that *clbB* mRNA expression was highest in anoxic cultures and decreased with
139 increasing oxygen concentration (Figure 5). Together with the observation that DNA cross-
140 linking activity and colibactin autotoxicity are inhibited by oxygen, these results demonstrated
141 that oxygen constrains colibactin production.
142
143 **ArcA is required for full DNA-crosslinking by *E. coli* producing colibactin**
144 We next sought to investigate the mechanism by which oxygen regulates colibactin production.
145 The ArcAB two component system, composed of the ArcB sensor and the response regulator
146 ArcA, is well known to sense microaerobic and anaerobic conditions (25). To test for a role in
147 colibactin production, we constructed a SP15 strain mutated for *arcA* and assessed its DNA
148 cross-linking activity at a low (0.1%) oxygen concentration (Figure 6). The DNA cross-linking
149 activity of the *arcA* mutant was significantly reduced compared to the wild-type strain, and was
150 restored in the mutant hosting a plasmid-encoded *arcA* gene (Figure 6A and B). The growth of
151 *E. coli* wild-type strain SP15, *arcA* mutant and complemented mutant strain was similar (Figure
152 6C), indicating that the altered cross-linking activity of the *arcA* mutant was not due to a growth
153 defect and reduced bacterial numbers. Thus, ArcA positively regulates colibactin production.
154
155 **Oxygen and *arcA* mutation inhibit the genotoxicity of *pks*+ *E. coli* infecting epithelial cells**
156 To confirm that oxygen inhibits colibactin production, we examined the genotoxicity of the *E.*
157 *coli* strain SP15 in epithelial cells grown in a standard atmosphere (21% O_2) or in "physioxic"
158 conditions (4% O_2) (32). Epithelial HeLa cells were infected 4 hours with the bacteria, at 21%
159 or 4% O_2, then DNA damage was visualized 4 hours later by immunofluorescence staining of
160 histone H2AX S139-phosphorylation (γH2AX) (1) (Figure 7). Infection with SP15 at 21%
161 oxygen inflicted detectable, low-level DNA damage, while infection at 4% oxygen induced

markedly higher DNA damage (Figure 7AB). HeLa cells treated with genotoxic cisplatin under 21% or 4% oxygen conditions had similar γH2AX levels, indicating that a modulation of the DNA damage response by oxygen cannot account for the increased genotoxicity of SP15 at 4% oxygen (Supplementary figure 1). We also assessed the genotoxicity of the *arcA* mutant in physioxic conditions. The mutant induced significantly reduced DNA damage, while the complemented mutant had its genotoxicity partially restored (Figure 7AB). Our data altogether show that oxygen concentration modulates colibactin production and genotoxic activity of *pks*+ *E. coli* on epithelial cells, via an *arcA*-dependent mechanism.

Discussion

We show that oxygen reduces the production and genotoxicity of colibactin, and that the Arc system which sense oxygen availability positively regulates colibactin production. This regulation of colibactin production is consistent with the observation that fitness and virulence systems of enterobacteria are usually regulated by oxygen (25). 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. Besides the intestinal niche, in the host epithelial tissues and fluids (such as urine), pathogenic enterobacteria encounter “physioxic” oxygen levels (2% to 9%, averaging ~4%) (32, 34). Pathogenic enterobacteria such as *Shigella*, enterotoxigenic *E. coli*, avian and uropathogenic *E. coli* tightly regulate the expression of their virulence factors in relation to oxygen concentration (35–38). For *pks*+ *E. coli*, the regulation of colibactin production by oxygen via the Arc system could be viewed as a selected advantage in relation with the regulation of virulence and fitness within the host. 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 (39).

194 As colibactin is susceptible to aerobic oxidation (2), an energy saving strategy that inhibits its
195 synthesis under environmental oxygen conditions leading to its inactivation may have been
196 selected. Alternatively, the adaptation of colibactin bioactivity and production to a low-oxygen
197 environment could be associated to the role of colibactin in the competition with the
198 polymicrobial community in the anoxic intestinal lumen. Indeed, recent findings highlighted a
199 direct role for colibactin in killing or inhibiting enteric bacteria (such as the anaerobe
200 *Bacteroides fragilis*), in the induction of prophages in polymicrobial enteric anerobic cultures,
201 and ultimately in shaping the gut microbiota (9, 10, 30). Our finding that an anoxic environment
202 is most favorable for colibactin production is thus consistent with the recently proposed role of
203 colibactin in interbacterial competition in gut lumen.

204 The notion that oxygen regulates colibactin production is also relevant to the question
205 of whether, where and when colibactin inflicts DNA damage in the gut. It has been repeatedly
206 reported that DNA damage was inflicted in enterocytes of rats and mice colonized or force-fed
207 with different strains of *E. coli pks*+ (NC101, 192PP, M1/5, SP15, 11G5, the probiotic strain
208 Nissle 1917) (13, 15, 16, 18, 40, 41). Keeping in mind that colibactin production is also
209 regulated by a variety of environmental cues such as iron, polyamines, and carbon sources (20–
210 22), the physiological hypoxia at the surface of the epithelium could constitute a favorable
211 environment for colibactin production and activity. Consistent with this hypothesis, we
212 observed in this work that *E. coli* SP15 induced marked DNA damage under physioxic
213 conditions. Other low oxygen intestinal compartments could favor even more colibactin
214 production, such as the stem cells that reside in a hypoxic niche (as low as 1% O_2) at the bottom
215 of the crypt (42). Signs of long-term persistent chromosomal instability have been reported in
216 animals colonized with *pks*+ *E. coli* (40) and the mutational signature of colibactin has been
217 found in intestinal crypts in healthy human subjects (43), suggesting that *pks*+ *E. coli* could
218 leave their genotoxic imprint in stem cells. As far as is described in the literature, *E. coli pks*+
219 have not yet been observed in the gut stem niche, but were shown to form biofilms together
220 with the anaerobe *B. fragilis* on the colonic epithelium of colorectal cancer patients (44). It is
221 well known that tumor tissue is hypoxic compared to healthy tissue (27), and thus could present
222 another favorable environment for colibactin production. It is also interesting to note that
223 inflammation appears to be a key factor in promoting colorectal tumorigenesis by *E. coli pks*+
224 in mouse models (17, 18). Severe inflammation can lead to local oxygen depletion, resulting
225 from tissue necrosis and neutrophil respiratory burst (26). Such hypoxic areas can also be
226 generated during infectious processes, when the host responds by coagulation to prevent
227 bacterial dissemination, thus generating a rapid drop in local oxygen tension (45). Hypoxia at

228 the level of the inflamed and infected tissues could therefore present environments favorable
229 for colibactin production. Further study of the localization of *pks*+ bacteria in relation to host
230 cell DNA damage and tissue inflammation will be necessary to better understand the impact of
231 colibactin on infection and cancer.

232 In conclusion, we provide evidence that oxygen decreases colibactin synthesis by *E. coli*
233 via an *arcA*-dependent regulatory mechanism, raising the hypothesis that colibactin production
234 and genotoxic activity are adapted to the bacterial warfare in the anoxic gut and also to hypoxic
235 infected, inflamed and tumor tissues.

236

237

238

239 **Materials and Methods**

240

241 **Bacterial strains, mutants, plasmids and primers**

242 The bacterial strains, plasmids and primers used in this study are listed in Table 1. Mutagenesis
243 of *arcA* gene was performed by using the lambda red mutagenesis method, and confirmed by
244 PCR, with the primers listed in Table 1. The mutant was trans-complemented with the pGEN-
245 arcA plasmid.

Strains, plasmids, primers	**Genotype, use, sequence**	**Reference**
Strains		
E. coli DH10B pBACpks	DH10B strain carrying the *pks*-island on a pBeloBAC11 vector	(1)
DH10B pBACpks *clbS*	*clbS::FRT* mutant	(7)
E. coli SP15	Clinical *E. coli* O18:K1 *pks*+ strain, isolated from the spinal fluid of a neonate with meningitis	(16)
E. coli SP15 *arcA*	*arcA::kan* mutant	This study

Plasmids			
pClbS	Plasmid pASK75 carrying *clbS*		(7)
pCM17	Plasmid containing the *luxCDABE* operon driven by a constitutive promoter		(46)
pMT3	pCM17-derived plasmid containing the *luxCDABE* operon driven by the *clbB* promoter region		(31)
pGEN-arcA	Plasmid pGEN-MCS carrying *arcA*		(38)
Primers			
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
PB60	Lambda red mutagenesis of *arcA*	5'-GGGACTTTTGTACTTCCTGTTTC GATTTAGTTGGCAATTTAGGTAGCAA ACGTGTAGGCTGGAGCTGCTTCG-3'	This study
PB61	Lambda red mutagenesis of *arcA*	5'-GAAAAATAAAAACGGCGCTAAAA AGCGCCGTTTTTATTGACGGTGGTAAA GCCGACATATGAATATCCTCCTTAGT -3'	This study
PB62	Verification of *arcA* mutant	5'-GCATCAGGCAGGTCAGGGAC-3'	This study
PB63	Verification of *arcA* mutant	5'-CCAGTAAAGAAGTTACCATGG-3'	This study

246

247 **Bacterial culture conditions**

248 The day before the experiments, the *E. coli* strains were grown overnight in a standard incubator
249 at 37°C with 240 RPM shaking in Lennox L broth (LB, Invitrogen) supplemented when
250 required with appropriate antibiotics (Kanamycin 50 µg/ml, streptomycin 50 µg/ml,
251 chloramphenicol 25 µg/ml or carbenicillin 50 µg/ml). Then, the bacteria were pre-cultured in
252 LB or in Dulbecco's modified Eagle's medium (DMEM) with 25 mM HEPES (Invitrogen) to
253 reach the exponential phase (OD600 ~ 0.4). Cultures at various oxygen concentrations were
254 performed in a hypoxic workstation (Whitley H35) regulated at 0.1% to 13% O_2, 5% CO_2, 37°C
255 and 70-90% humidity. To achieve a 0% O_2 anoxic atmosphere, we used a jar with anaerobic
256 atmosphere generation bags (Sigma) manipulated within the hypoxic workstation set at 0.1%
257 oxygen. Standard microbiological and cell culture incubators were used for the 21% O_2
258 (atmospheric) condition. Culture media were equilibrated for 16-24 h at the required oxygen
259 concentration before use. For DH10B strains (Figure 1), LB cultures were inoculated with
260 $2x10^6$ bacteria/ml in 5 ml and grown overnight (17 h) in fully closed 10 ml tubes (Kima), or in
261 opened 50 ml tubes (Falcon), or in 14 ml 17x100 mm tubes with the cap in vented position
262 (Falcon 352059). The *E. coli* strain SP15 was inoculated ($1.5x10^7$ bacteria) in 1 ml DMEM
263 Hepes in Falcon 352059 tubes with the cap in vented position and cultivated 3.5 h with 240
264 RPM agitation at 37°C within the hypoxystation set at the oxygen concentration required. For
265 CFU enumeration, the cultures were serial-diluted in PBS, plated on LB agar plates and
266 incubated overnight at 37°C in a standard incubator.
267
268 **Exogenous DNA cross-linking by colibactin-producing *E. coli***
269 The assay was performed as described before (3). Briefly, 100µl of bacterial culture were added
270 with 500 ng DNA (pUC19 linearized by BamHI digestion) and 1mM EDTA, then incubated 40
271 min at the indicated oxygen concentration. The DNA was recovered using a Qiagen PCR
272 purification kit, analyzed by denaturing gel electrophoresis, stained with GelRed (Biotium) and
273 visualized with a Bio-Rad Chemidoc XRS system. DNA bands were quantified from
274 unsaturated flat-fielded images using ImageJ.
275
276 **Quantification of N-myristoyl-D-AsnOH (C14-Asn)**
277 Bacterial supernatants (1.5 ml) were prepared by centrifugation at 5000 g, filtration through
278 0.22 µm PVDF filters (Millipore) and stored at -80°C. Lipids were extracted and analyzed as
279 before (47): briefly, 1 ml of sample was extracted on solid phase 96 wells plates (OASIS HLB,
280 Waters), eluted with methanol, evaporated twice under N_2 then suspended in 10 µl methanol.
281 The quantification of C14-Asn was performed by the MetaToul Lipidomics facility (Inserm

282 UMR1048, Toulouse, France), using an in-house quantification assay by high-performance
283 liquid chromatography/tandem mass spectrometry analysis.
284
285 **Bioluminescence monitoring of *clbB* promoter activity**
286 The *E. coli* strain SP15 hosting pMT3 or pCM17 (containing the *Photorhabdus luminescens*
287 *luxCDABE* operon driven by the *clbB* promoter region (31) or a constitutive promoter (46)
288 respectively) was cultivated at the required concentration of oxygen, then 100µl were
289 transferred on 96 wells black plates (Greiner), the plate was shaken vigorously 5 sec to provide
290 oxygen to the luciferase and the bioluminescence was measured with a Tecan Spark plate
291 reader, or photographed with a Bio-Rad Chemidoc XRS system.
292
293 ***clbB* mRNA quantification**
294 10^9 bacteria were collected by centrifugation and stored at -80°C. RNA was purified using the
295 RNA Easy plus mini kit (Qiagen), then 2 µg were used to synthesize cDNA with the i-script kit
296 (Bio-Rad). For standard range of *clbB* and *cysG* housekeeping gene (48), PCRs were performed
297 using *E. coli* SP15 genome as a template and the primer pairs NG49/NG50 and NG53/NG54
298 respectively. Standards were purified and serial diluted from 5.10^9 to 5.10^1 copy/µl. Real time
299 PCR were performed with the iQ SYBR Green supermix (Bio-Rad), with 2 µl of cDNA or
300 standard dilutions, and 187.5 nM of primer pairs NG61/NG62 (*clbB*) or NG55/NG56 (*cysG*).
301 The copy numbers of *clbB* and *cysG* were calculated from the Ct obtained using the standard
302 curves. The expression of *clbB* gene at different oxygen concentration was calculated by the
303 ratio *clbB* / *cysG* copy numbers and normalized at 100% by the mean at 0.1% oxygen.
304
305 **Epithelial cells infection and quantification of DNA-damage**
306 The assay was performed as described before (3) with some modifications. HeLa cells were
307 cultivated in a standard atmospheric 37°C 5% CO_2 incubator, in DMEM Glutamax (Invitrogen)
308 supplemented with 10% fetal calf serum (FCS), 50 µg/ml gentamicin and 1% non-essential
309 amino acids (Invitrogen). The cells were seeded ($2.5x10^4$ cells/well) in 8-chambers slides
310 (Falcon) and grown 24 hours in the atmospheric 5% CO_2 incubator, or in the hypoxic
311 workstation set at 4% O_2 5% CO_2. The cells were infected in DMEM 25 mM HEPES at a
312 multiplicity of 100 bacteria per cell and incubated 4 hours in the hypoxic or standard incubator.
313 As controls, the cells were left uninfected or were treated with 20 µM cisplatin (Sigma) to

induce DNA-damage. The cells were then washed 3 times with HBSS supplemented with 100 µg/ml gentamicin, incubated in complete cell culture medium supplemented with 100 µg/ml gentamicin for 4 hours, in the same hypoxic or standard incubator. The cells were finally washed in PBS and fixed 15 min in PBS 4% formaldehyde. Following 5 min permeabilization in 0.2% Triton X-100, and blocking in MAXblock medium (Active Motif), the cells were stained with antibodies against γH2AX (1:500, JBW301, Millipore) followed with anti-mouse AlexaFluor 488 (1:1000, Invitrogen). The cells were mounted in Fluoroshield w/DAPI medium (Sigma), and examined with a Zeiss 710 laser scanning confocal microscope at 63X. The mean fluorescence intensities of γH2AX within the nuclei were analyzed using a NIH ImageJ macro: the nuclei were identified in the DNA image and the mean fluorescence intensity of γH2AX was measured in the masked green channel for each nucleus.

Statistical analyses

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

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Disclosure statement

The authors report there are no competing interests to declare.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article.

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508 **Figures**
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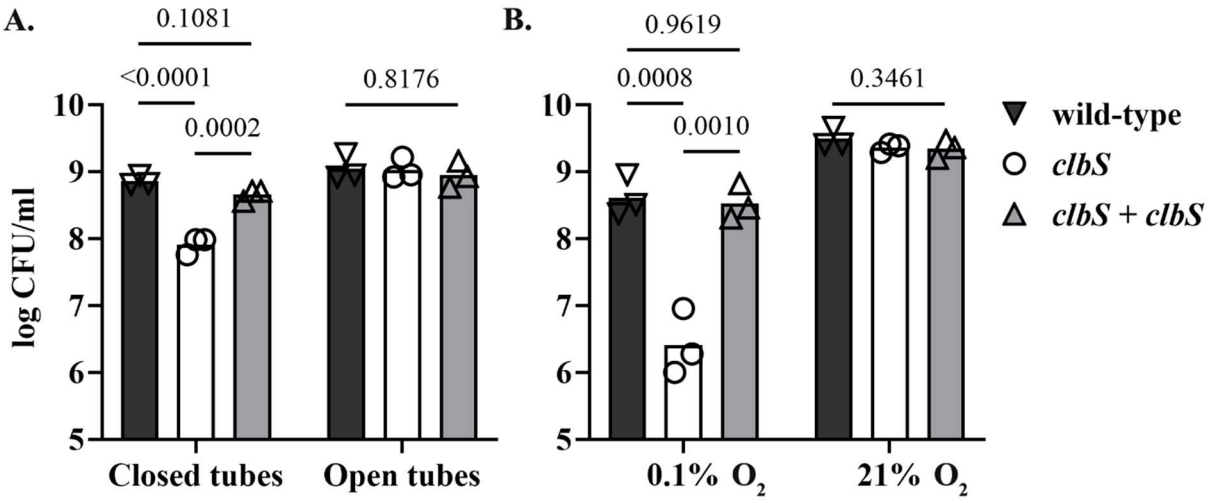

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513 **Figure 1: Culture oxygenation modulates the autotoxicity phenotype of a *clbS* mutant in**
514 **a *pks*+ *E. coli*.**
515 **A**. *E. coli* strain DH10B pBAC*pks*, the isogenic *clbS* mutant deficient for colibactin self-
516 resistance protein, and the trans-complemented *clbS* mutant (*clbS* + *clbS*), were grown (5 ml
517 LB) with shaking in a standard atmospheric incubator, in 10 ml tubes that were tightly closed,
518 or in 50 ml tubes with the cap opened. **B.** The bacteria were cultured with shaking in tubes with
519 cap in vented position, within a hypoxic chamber at 0.1% O_2, or in a standard atmosphere
520 incubator (21% O_2).
521 After 16 h culture, bacterial growth was determined by plating and counting colony forming
522 units (CFU). The mean and individual results of three independent experiments are shown, with
523 the p values of an ANOVA and Tukey's multiple comparison test.
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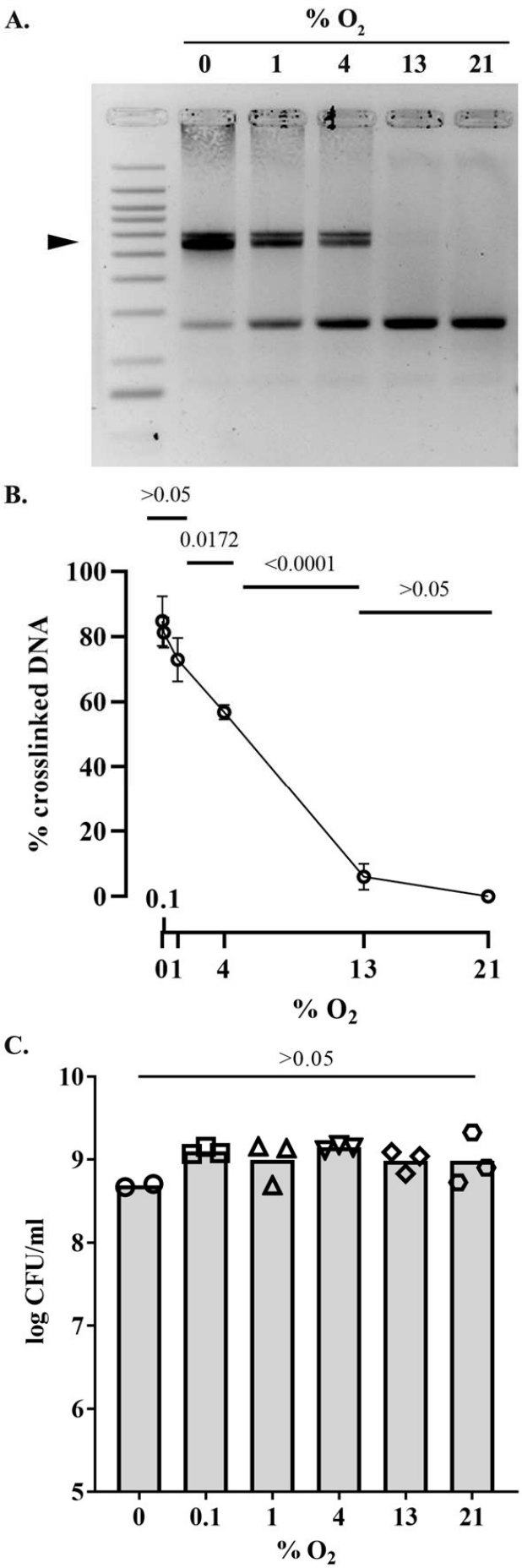


Figure 2: Effect of oxygen concentration on the DNA crosslinking activity of a *pks*+ *E. coli.*

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).

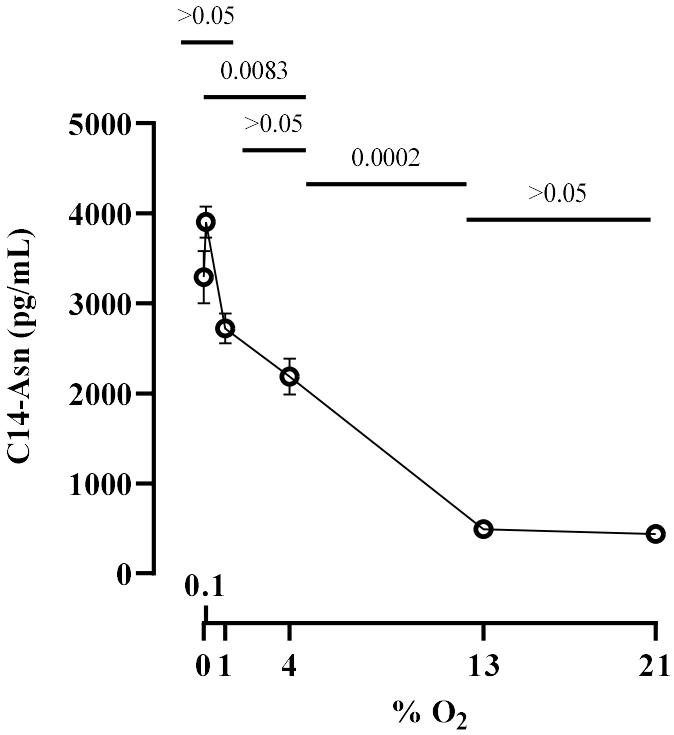


Figure 3 : Effect of oxygen concentration on the production of colibactin cleavage product C14-Asn.

The *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.

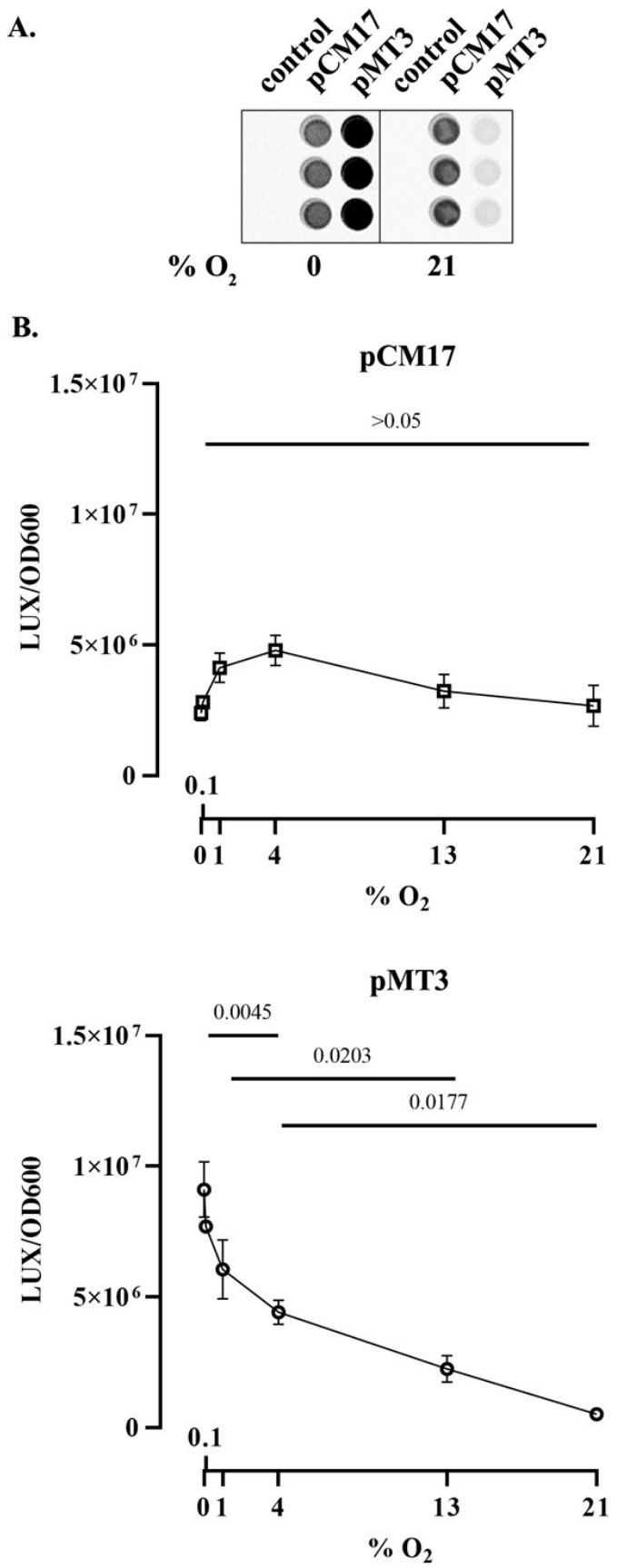


Figure 4 : Effect of oxygen concentration on *clbB* promoter activity.

A. Luminescence of *E. coli* strain SP15 carrying the luciferase reporter plasmid pCM17 or pMT3 (constitutive promoter or *clbB* promoter upstream of *luxCDABE* respectively), or the plasmid without promoter as a control. Three independent bacterial cultures grown 3.5 h at 0% or 21% 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 provide the oxygen required to the reaction catalyzed by the luciferase.

B. The luminescence (normalized to the 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.

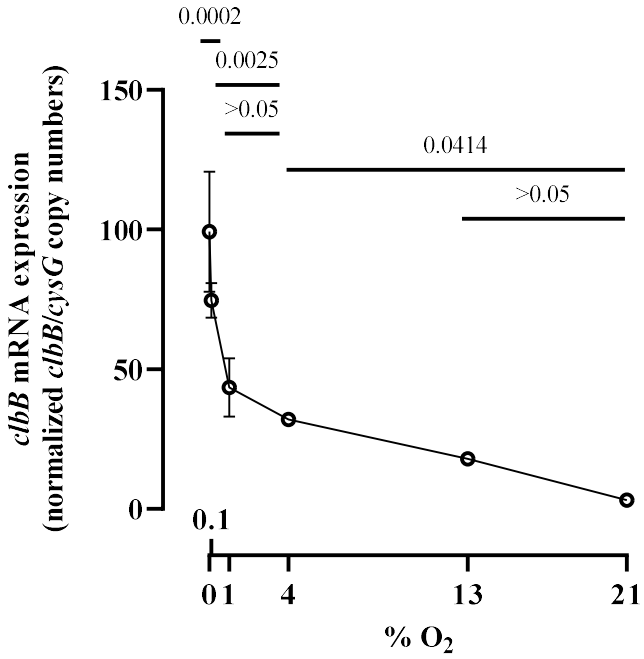


Figure 5 : Effect of oxygen concentration on *clbB* gene expression.
The mRNA levels of *clbB* gene were determined in *E. coli* SP15 grown 3.5 hours at specified percentages of oxygen. The DNA copy number of the *clbB* mRNA relative to that of the *cysG* housekeeping gene was normalized to the mean 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.

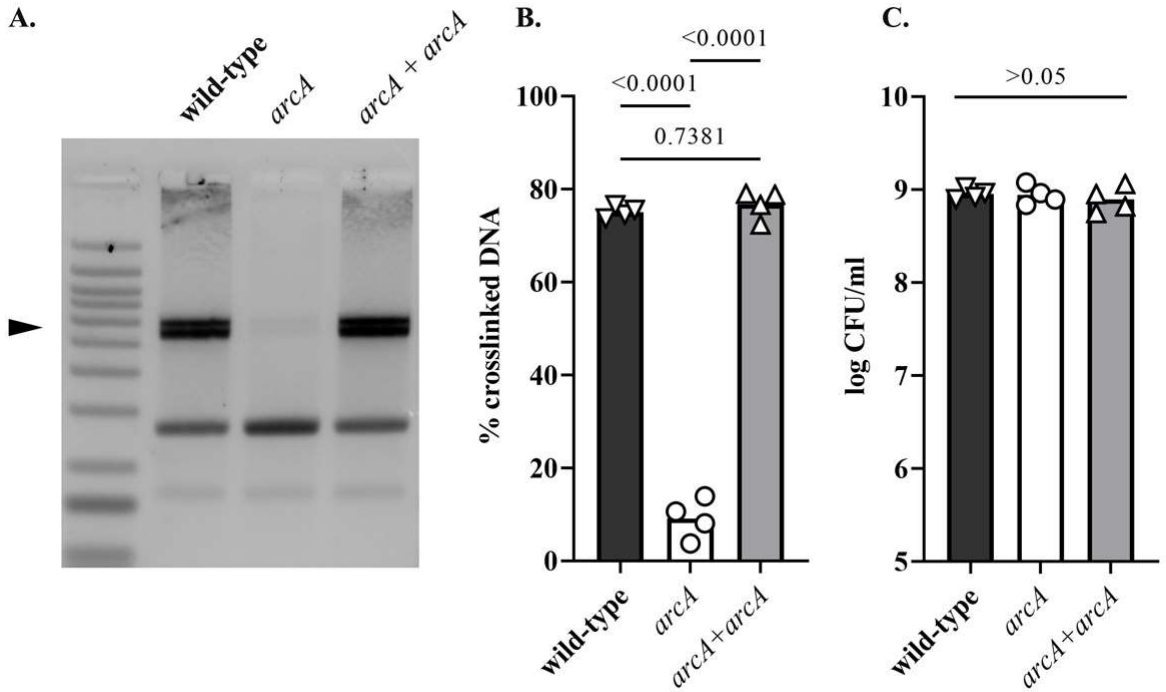


Figure 6: Deletion of *arcA* results in decreased DNA crosslinking activity of a *pks*+ *E. coli.*
A. *E. coli* wild-type strain SP15, *arcA* mutant and *arcA* mutant complemented with a plasmid-encoded *arcA* were grown 3.5 hours at 0.1% oxygen, then incubated 40 min with linearized plasmid DNA. The DNA cross-linked by colibactin with apparent doubling in size (arrow) was visualized by denaturing gel electrophoresis.
B. The percentage of DNA signal in the crosslinked DNA band relative to the total DNA signal in the lane was determined by image analysis. The results of four independent cultures 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 was examined by plating and counting colony forming units (CFU).

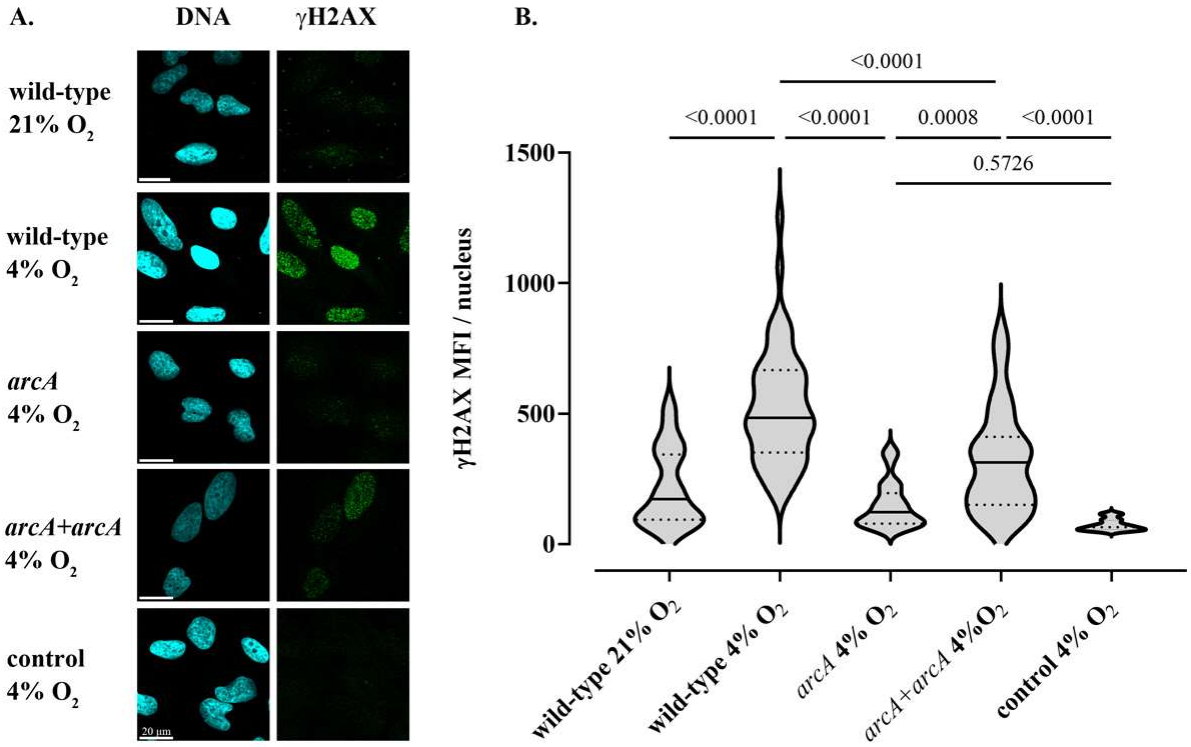
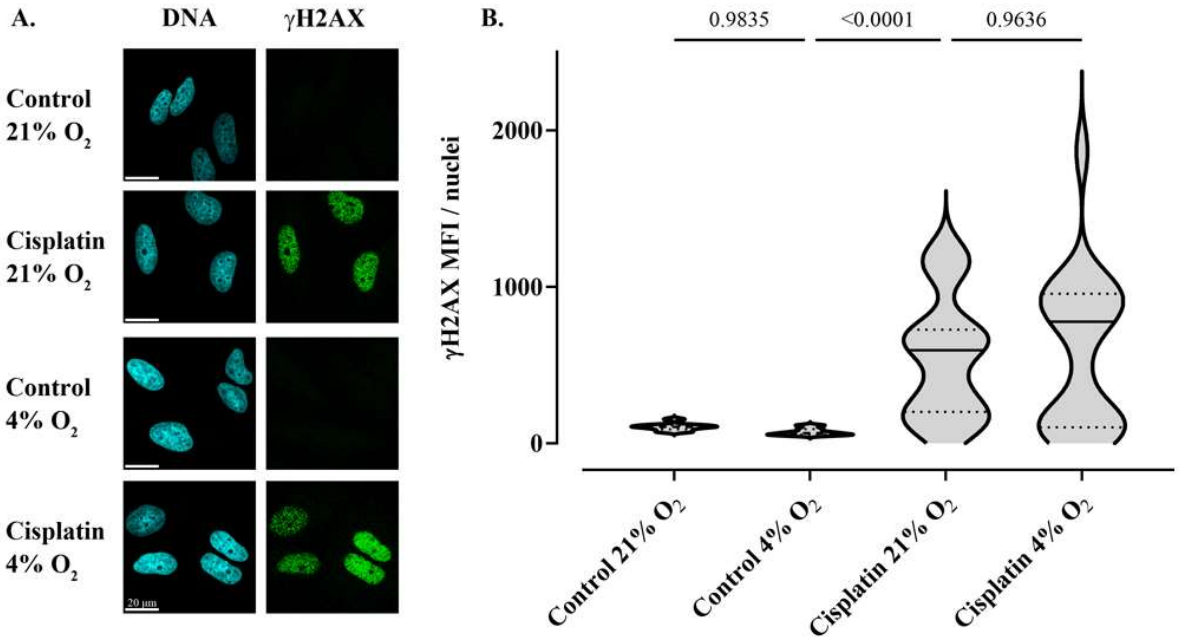


Figure 7: Oxygen and the *arcA* mutation inhibit the genotoxicity of colibactin-producing *E. coli*.

A. HeLa epithelial cells were infected with *E. coli* wild-type strain SP15, the *arcA* mutant, or the *arcA* mutant complemented with plasmid-encoded *arcA,* for 4 hours under 21% or 4% oxygen atmosphere, and then cellular DNA-damage was demonstrated by γH2AX immunofluorescent staining. DNA was counterstained with DAPI. Scale bar = 20 µm

B. γH2AX mean fluorescent intensity (MFI) within ~50 cell nuclei was measured by image analysis. The violin plots show the frequency distribution of the data, with the median and quartiles as solid and dotted lines. P values were calculated with an ANOVA and Tukey's multiple comparison test.



Supplementary figure 1: Oxygen does not alter the γH2AX response of genotoxin-treated HeLa cells.

A. HeLa epithelial cells were treated with cisplatin for 4 hours under 21% ("hyperoxic") or 4% ("normoxic") oxygen atmosphere, and then cellular DNA-damage was demonstrated by γH2AX immunofluorescent staining. DNA was counterstained with DAPI. Scale bar = 20 µm

B. γH2AX mean fluorescent intensity (MFI) within ~50 cell nuclei was measured by image analysis. The violin plots show the frequency distribution of the data, with the median and quartiles as solid and dotted lines. P values were calculated with an ANOVA and Tukey's multiple comparison test.