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EHEC/EAEC O104:H4 strain linked with the 2011 German outbreak of haemolytic uremic syndrome enters into the viable but non-culturable state in response to various stresses and resuscitates upon stress relief

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Various non-spore forming bacteria, including Escherichia coli, enter a dormant-like state, the viable but non-culturable (VBNC) state, characterized by the presence of viable cells but the inability to grow on routine laboratory media. Upon resuscitation, these VBNC cells recover both culturability and pathogenicity. In 2011, a large outbreak involving more than 3000 cases of bloody diarrhoea and haemolytic uremic syndrome was caused by an E. coli O104:H4 strain
expressing genes characteristic of both enterohaemorrhagic (EHEC) and enteroaggregative E. coli (EAEC). The ability of the outbreak strain to enter the VBNC state may have complicated its detection in the suspected sources. In this paper, we investigated the ability of the outbreak strain to enter and subsequently recover from the VBNC state.We found that in a nutrient-poor micro-environment, various stresses such as toxic concentrations of copper ions or certain types of tap water are able to render the bacteria unculturable within a few days. Without copper ion stress, the majority of cells remained culturable for at least 40 days. Incubation with the stressors at 23°C compared with 4°C hastened this observed loss of culturability. The integrity of a considerable fraction of copper ion- and tap water 1-stressed bacteria was demonstrated by live/dead staining and microscopy. Relieving stress by copper-ion chelation facilitated resuscitation of these bacteria while preserving their fitness, major virulence gene markers (stx2, aggR, aggA genes) and specific phenotypes (ESBL resistance, autoaggregation typical for EAEC strains).

Introduction

In 2011 an enterohaemorrhagic (EHEC)/enteroaggregative *Escherichia coli* (EAEC) hybrid strain of serotype O104:H4 expressing genes for Shigatoxin 2a (*stx2a*), AAF/I fimbriae (plasmid-coded) and extended spectrum β-lactamase resistance (ESBL) (plasmidcoded) caused a large outbreak of haemolytic uremic syndrome (HUS) and bloody diarrhoea in northwestern Germany (Askar *et al*., 2011; Bielaszewska *et al*., 2011; Brzuszkiewicz *et al*., 2011; Cui *et al*., 2011; Frank *et al*., 2011; Mellmann *et al*., 2011; Scheutz *et al*., 2011). From early May through July 26th 2011, 3052 cases of EHEC diarrhoea and 733 cases of HUS, which resulted in the deaths of a total of 45 people, were reported to the German public health authorities (RKI, 2011a).

Whereas *E. coli* O157:H7 or O157:H-(NM) are the primary EHEC serotypes associated with development of HUS and bloody diarrhoea, EAEC are strongly linked to diarrheal illness in Africa, Latin America, Asia, but also in Europe and North America (Adachi *et al*., 2001; Karch and Bielaszewska, 2001; Kaper *et al*., 2004; Harrington *et al*., 2005; Tarr *et al*., 2005; Huang *et al*., 2006; Pennington, 2011). Until the year 2011 only a few EHEC/EAEC O104:H4 strains have been isolated from patients (Scheutz *et al*., 2011). One of these is the EHEC/EAEC strain HUSEC041 isolated from a HUS patient in 2001, which is genetically related to the recent outbreak strain with some important differences, such as its encoding of AAF/III instead of AAF/I fimbriae and its lack of ESBL resistance (Mellmann *et al*., 2008; 2011).

Several lines of evidence point to fenugreek sprouts and seeds as culprit sources of contamination (BfR+BVL+RKI, 2011; Gault *et al*., 2011; RKI, 2011b). However, the outbreak strain was detected in suspect food only in a small minority of cases, for example in an already opened package of sprouts from a household with disease (BfR, 2011). With respect to the fenugreek seeds from suspect origins, neither detection nor isolation of the *E. coli* O104:H4 outbreak strain has been possible thus far. The failure to isolate the EHEC/EAEC outbreak strain may have several reasons. For example, the bacteria may exist in the environment in a dormant state, such as the viable but non-culturable (VBNC) state, and detection by culture-based approaches may be possible only under certain conditions.

Many different bacterial species, including *E. coli*, are known to enter the VBNC state when under stressful environmental conditions, such as lack of nutrients, unfavourable temperatures or, the presence of toxic metal ions (Kell *et al*., 1998; Barer and Harwood, 1999; Oliver, 2005; 2010). Although alive and metabolically active at a low level, bacteria in the VBNC state do not grow on bacteriological media in which they would normally grow under a culturable state (Rahman *et al*., 1994). VBNC cells may regain culturability when resuscitated, which in some cases is driven by stress relief (Xu *et al*., 1982; Wingender and Flemming, 2011). VBNC cell formation has been shown for non-pathogenic *E. coli* but also for EHEC O157:H7 (Xu *et al*., 1982; McKay, 1992; Mizunoe *et al*., 1999; Grey and Steck, 2001; Yaron and Matthews, 2002; Pinto *et al*., 2011). Numerous VBNC-inducing factors have been reported; with respect to *E. coli,* these include nutrient starvation (Smith *et al*., 1994; Pinto *et al*., 2011), and high copper-ion concentrations (Grey and Steck, 2001). Once VBNC state cells are established, they may persist for long periods of time. Survival of *E. coli* O157:H7 for several months in water or soil environments, even in conditions of dryness that were initially thought to be critical, has been observed (Wang and Doyle, 1998; Bolton *et al*., 1999; Jiang *et al*., 2002).

Although the pathogenicity of VBNC cells *per se* is controversial, there is irrefutable evidence that recovered cells regain virulence, for example by animal passage, and may cause disease (Colwell *et al*., 1985; Rollins and Colwell, 1986; Stern *et al*., 1994; Oliver and Bockian, 1995). Nevertheless, even continuous Shiga-like toxin production or s*tx1* gene expression has been shown for VBNC state EHEC O157:H7 (Yaron and Matthews, 2002; Liu *et al*., 2010). Recovery of virulence after resuscitation was demonstrated in a range of microorganisms, such as *Vibrio cholerae, Campylobacter jejuni* (Jones *et al*., 1991; Saha *et al*., 1991)*, Legionella pneumophila* (Hussong *et al*., 1987; Steinert *et al*., 1997) and *Shigella dysenteriae* (Rahman *et al*., 1994; 1996; Colwell *et al*., 1996). The VBNC phenomenon may therefore represent a health and surveillance problem due to the almost universally used culture-based pathogen detection methods.

Because the German *E. coli* O104:H4 outbreak strain represents a novel hybrid bacterium composed of genes characteristic of EHEC and EAEC (Askar *et al*., 2011; Bielaszewska *et al*., 2011; Brzuszkiewicz *et al*., 2011; Cui *et al*., 2011; Frank *et al*., 2011; Mellmann *et al*., 2011; Scheutz *et al*., 2011), it is difficult to extrapolate on its dormant state behaviour from what has been observed with either EHEC (see above) or EAEC. However, in the case of EAEC, to our knowledge no data on VBNC formation are available until yet. Therefore, we tested here the response of the outbreak strain to various sublethal stresses (nutrient deprivation, copper stress, temperature change) and observed its behaviour in different types of tap water. We showed that the EHEC/EAEC O104:H4 outbreak strain does indeed enter a VBNC state in response to various stresses and resuscitates to become a potentially pathogenic bacterium upon stress relief.

Results

Culturability of E. coli *O104:H4 severely decreased after incubation with copper ions or tap water 1 within a few days*

To investigate *E. coli* O104:H4 2011 outbreak strain persistence in different water microcosms, about 10^6 bacteria per millilitre were inoculated into different nutrient-poor microcosms and the kinetics of culturability was determined. Those conditions were 0.9% saline (PK), 0.9% saline containing 500 µM CuSO4 (PK + Cu^{2+}), and tap water from two different German regions (town 1 and town 2, i.e. tap 1 and tap 2, see Fig. S1 for water analysis). We included copper ion-mediated stress conditions because several authors have reported that elevated but sublethal concentrations of copper ions can induce a VBNC state in bacteria and/or that resuscitation was possible after stress relief, e.g. in non-pathogenic *E. coli* (Grey and Steck, 2001), *Pseudomonas aeruginosa* (Dwidjosiswojo

et al., 2011), or *Agrobacterium tumefaciens* and *Rhizobium leguminosarum* (Alexander *et al*., 1999). Because low temperatures promote survival and culturability of bacteria (Mizunoe *et al*., 1999; Grey and Steck, 2001; Ogden *et al*., 2001; Pinto *et al*., 2011), all conditions were tested at ambient temperature (23°C) and at 4°C. We found that culturability markedly differed between the starvation solutions and the temperature used with the expected general trend to recover more cfu at 4°C (Table 1). Bacteria were able to persist and to retain culturability over a period of at least 40 days when incubated in 0.9% saline, independent of the temperature. Incubations with tap 2 showed culturability over 40 days with a better bacterial recovery at 4°C (Table 1). In contrast, when additional stress factors (copper ions or tap 1) were present in the microcosms at 23°C, culturability rapidly decreased. Zero cfu were determined after 3 days in 0.9% saline + Cu^{2+} or tap 1 (Table 1). Among samples incubated at 4°C, reduction of culturability progressed more slowly; zero cfu were obtained after 5 days in 0.9% saline + Cu^{2+} and were almost obtained after 40 days in tap 1 (Table 1). Zero cfu states were verified by plating of 1 ml culture at all shown time points after entry into unculturability as well as at the time points used for resuscitation experiments. In those cases not a single colony grew on the agar even when the plates were incubated for 7 days to allow retarded bacterial growth (data not shown).

Copper ion- or tap 1-stressed non-culturable E. coli *O104:H4 populations contain viable bacteria*

To test whether the unculturable microcosms obtained by starvation and copper-ion or tap 1 stress are unculturable due to bacterial death or loss of colony-forming ability, we performed propidium iodide (Pi)/Syto9 live/dead staining (Boulos *et al*., 1999; Stocks, 2004) (Fig. 1). The fraction of membrane-damaged cells at 4°C increased from day 10 to day 40 from 25% to 44% respectively, and was about 95% at day 40 in the copper ion-stressed microcosm at 23°C (Fig. 1A and C). In the completely or almost completely unculturable tap 1 microcosms incubated for 40 days at 4°C or 23°C, about 77% or 17% of the bacteria had intact membranes respectively (Fig. 1B and C). It was further obvious especially in the incubations with a high proportion of membrane-damaged cells (PK + Cu^{2+} at 23°C and tap 1 at 23°C) that the viable bacteria were associated with microaggregates (Fig. 1A and B). In summary, our results show that even in microcosms where no cfu was recovered for more than 30 days, a fraction of the *E. coli* O104:H4 outbreak strain preserved intact cell membranes and therefore was apparently viable.

Resuscitation of copper ion-induced VBNC cells by stress relief and conservation of strain characteristics and fitness after resuscitation

The observation of apparently living bacteria within unculturable populations implied that a proportion of these cells exists in the VBNC state (Xu *et al*., 1982; Rollins and Colwell, 1986; Kell *et al*., 1998; Oliver, 2005). Therefore, we assessed whether the unculturable bacteria reassumed a reproductive state, a procedure commonly termed resuscitation. Successful recovery of stress-induced non-pathogenic *E. coli* and EHEC O157:H7 VBNC bacteria into a culturable state was achieved earlier by stress removal, such as relief of Cu^{2+} stress by the means of chelators (Grey and Steck, 2001; Liu *et al*., 2009). VBNC cells produced by various stresses were only able to resuscitate for a limited time and when they entered the VBNC state at 4°C (Pinto *et al*., 2011). Therefore, we focused on resuscitating the 4°C copper ion-stressed microcosm after 6 and 11 days of incubation, i.e. at days 1 and 6 after complete lack of culturable cells in 1 ml (cfu <1/1 000 000 cfu ml^{-1}). At those time points, the bacteria were repeatedly washed with cold EDTA and subsequently plated on rich agar medium. A fraction of about 60 cfu ml^{-1} was recovered by that procedure at day 1 and about 30 cfu ml^{-1} at day 6, showing that recovery of the *E. coli* O104:H4 outbreak strain into a culturable state was indeed possible (Fig. 2A). No culturable bacteria were recovered by repetitive washing of the bacteria without EDTA (data not shown).

Next, we verified whether six selected colonies exhibited the characteristics of the outbreak strain. First, the colonies all grew on ESBL selective media, due to the presence of the resistance plasmid carrying a CTX-M-15 gene (data not shown) (Askar *et al*., 2011; Bielaszewska *et al*., 2011; Brzuszkiewicz *et al*., 2011; Cui *et al*., 2011; Frank *et al*., 2011; Scheutz *et al*., 2011). Second, all six colonies yielded the characteristic PCR pattern for amplification of *stx2*, *terD*, *rfbO104* and *fliC H4* (Fig. 2B, data shown for three clones) (Bielaszewska *et al*., 2011). Third, all tested clones shared a comparable DNA macrorestriction pattern (PFGE profile) and plasmid profile with the parental strain, implying no major genetic changes and retention of both large plasmids [83 kbp EAEC virulence plasmid and 90 kbp ESBL antibiotic resistance plasmid (Mellmann *et al*., 2011)] after resuscitation (Fig. 2C and D, data shown for three clones). Fourth, genetic determinants for the aggregative phenotype, such as the *aggR* gene coding for the master regulator of the EAEC virulence plasmid and

chromosomal genes and the *aggA* (AAF/I) gene coding for the AAF/I fimbrial subunit were present in the resuscitated clones (Fig. 2E, data shown for three clones) (Okeke and Nataro, 2001; Nataro, 2005). Fifth, fitness of three recovered colonies compared with the parental strain, as judged by growth in liquid medium, proved identical (Fig. 2F). These data confirm the recovery of outbreak strain-derived colonies and support the claim that the recovered clones are still virulent, as illustrated by the presence of major virulence characteristics like the *stx2, aggR* and *aggA* genes.

Cell morphology of different E. coli *strains as well as parental, VBNC and resuscitated* E. coli *O104:H4*
We compared cell shapes and dimensions of the non-pathogenic *E. coli* K12 strain with three different EHEC strains, including the classical EHEC O157:H7, the 2011 German HUS outbreak *E. coli,* and HUSEC041, the latter two being serotype O104:H4 and EHEC/EAEC strains (Mellmann *et al*., 2008; 2011; Bielaszewska *et al*., 2011; Brzuszkiewicz *et al*., 2011; Scheutz *et al*., 2011). *E. coli* K12 exhibited the typical morphology of straight non-aggregating bacilli with length and diameter of about 2 μM and 0.6 μM respectively (Fig. S2A and D). The *E. coli* O157:H7 strain (mean length 1.5 μM and diameter 0.8 μM) and the two *E. coli* O104:H4 strains (mean length 1.4 μM and diameter 0.9 μM) were significantly shorter and thicker than *E. coli* K12 (Figs S2 and 3A). Only the two O104:H4 *E. coli* strains formed aggregative clusters (Figs S2C and 3A). We observed that broth-grown resuscitated colonies retained the autoaggregative phenotype, implying that the enteroaggregative virulence plasmid is also active in the VBNC revertant (Fig. 3C). We further noted a statistically significant decrease in length and diameter of the VBNC cells (length ~ 1.2 μM, diameter ~ 0.8) compared with both the parental cells and resuscitated cells (length ~ 1.4 μM, diameter ~ 0.9) (Figs 3 and S2).

Discussion

A number of pathogenic bacteria enter the VBNC state when stressed. We here determined that the *E. coli* O104:H4 isolate linked to the 2011 outbreak of bloody diarrhoea and HUS in Germany persists in a variety of nutrient-poor environments (0.9% saline and tap 2) for at least 40 days with only a minor reduction in cfu counts (Table 1). The outbreak strain responded to increasing stress with rapidly decreasing cfu counts, as in the case of copper ion-containing microcosms or cultures starving in tap 1 but not tap 2 (Table 1). Reduced temperature (4°C) supported survival of cells both in the culturable and the non-culturable state compared with the elevated temperature (23°C) (Table 1 and Fig. 1). This observation is consistent with data from other studies focusing on non-pathogenic *E. coli* and EHEC O157:H7 (Jiang and Chai, 1996;Wang and Doyle, 1998; Grey and Steck, 2001). The copper ion- or tap 1-stressed microcosms at 23°C and the copper ion-stressed incubations at 4°C reached the state of unculturability (cfu <1/1 000 000) after 3–5 days of starvation (Table 1). The 100% decrease in culturability was not reflected in the number of apparently living cells as demonstrated with Pi/Syto9 staining.

Tap 1 conferred obviously more pressure on the bacteria for entry into a non-culturable state than tap 2. The abiotic parameters determined according to the German Drinking Water Ordinance were almost comparable (Fig. S1). Interestingly, although its copper concentration was less than the concentrations used for the copper ion-stress conditions (PK + Cu^{2+} = 500 μM versus tap 1 = 1.2 μM), the copper ion concentration is at least about 15-fold higher in tap 1 than in tap 2 (0.079 versus <0.005 mg l^{-1}). As tap 1 but not tap 2 was disinfected with the addition of chlorine dioxide (0.2 mg l^{-1}) at the water purification plant before delivery to households (personal communication with water purification plants), it is possible that tap 1 contains trace amounts of hypochlorite or hypochlorous acid, despite their low stability.

In this work, we successfully resuscitated a fraction of apparently living but non-culturable cells within the copper ion-stressed microcosms at 4°C by relieving stress (Fig. 2A). The number of resuscitated bacteria decreased over time, and we were able to resuscitate cells 1 and 6 days after complete non-culturability was reached (Fig. 2A). Grey and Steck recovered copper-stressed *E. coli* O157:H7 up to 2 weeks after attaining nonculturability by using a similar EDTA washing approach (Grey and Steck, 2001). We further showed that phenotypic (cell morphology), virulence and other strain-specific characteristics, like *stx2, aggR* and *aggA* gene presence, ESBL resistance, and the aggregative phenotype, were preserved after resuscitation (Figs 2 and 3).

Our attempts to resuscitate viable cells originating from the 23°C copper ion-stressed microcosm as well as the microcosms stressed in tap 1 by using the EDTA washings and subsequent plating on non-supplemented, catalase-, or sodium pyruvate-supplemented rich agar failed even at early time points

(data not shown). Our inability to resuscitate bacteria stressed at 23°C is consistent with the results of others (Grey and Steck, 2001; Pinto *et al*., 2011). To improve the extent of resuscitated colonies after copper-ion stress and to establish an adequate recovery protocol for other stress conditions, such as the tap 1 incubations, identification of the exact entry mechanisms into the VBNC state and their stimuli will become necessary.

Interestingly, we noted that in incubations with high proportions of dead cells (copper-ion stress or tap 1 at 23°C), intact bacteria were enriched in microaggregates (Fig. 1). Autoaggregation is a characteristic phenotype of the EAEC pathovar driven by the presence of autoaggregative fimbriae coded on the pAA (plasmid of aggregative adhesion) virulence plasmid (Vial *et al*., 1988). Both the *E. coli* O104:H4 outbreak strain from 2011 as well as HUSEC041 from 2001 showed autoaggregation in liquid media but not *E. coli* K12 or classical EHEC O157:H7 (Figs 3A and S2), implying that the aggregative phenotype of EAEC might not only support its persistence in the human digestive tract (Vial *et al*., 1988; Pereira *et al*., 2008) but also may facilitate persistence in the environment. Comparison of the *E. coli* O104:H4 EHEC/EAEC outbreak strain genome with other *E. coli* genomes indeed showed that it is most closely related to the EHEC/ EAEC HUSEC041 strain and to the EAEC 55989 strain, isolated from an HIV-infected person suffering from persistent diarrhoea (Mossoro *et al*., 2002; Touchon *et al*., 2009; BGI, 2011; Brzuszkiewicz *et al*., 2011; Mellmann *et al*., 2011; Rohde *et al*., 2011)

Our study shows that the existence of VBNC state *E. coli* O104:H4 may be an additional factor to consider in successfully detecting this pathogen in an environmental or food source. In many cases, poor sensitivity of gene detection is due to low loads of the target bacterium in
food or environmental samples and/or the presence of PCR inhibitors that may co-purify with the target DNA (Gonzalez *et al*., 1999). Thus, enrichment of the target pathogen before PCR verification is necessary, which in the case of VBNC cells could lead to amplification of other bacterial DNA but likely not to resuscitation of the VBNC cell fraction. We therefore recommend taking into account the existence of VBNC bacteria for both culture-based as well as gene detection-based (PCR) protocols. In the future, it will therefore be important to identify the specific factors involved in triggering resuscitation of VBNC bacteria.

Experimental procedures

Bacterial strains and growth conditions
Escherichia coli strains K12 (C600), O157:H7 (EDL933), O104:H4 (RKI 01-09591), which is listed in the HUSEC collection as HUSEC041 (Mellmann *et al*., 2008), and O104:H4 (RKI 11-02027), were routinely grown at 37°C for 1 day on nutrient agar (NA) plates (Oxoid) unless otherwise stated, or when appropriate cultured in Luria–Bertani (LB) broth at 37°C with shaking (250 r.p.m.). The latter *E. coli* O104:H4 strain was isolated and characterized at the National Reference Centre for *Salmonella* and other Enteric Bacterial Pathogens (Robert Koch-Institut, Germany) within the 2011 German outbreak from a HUS patient and shows the typical outbreak strain markers: serotype O104:H4, *stx2* positive, *eae* negative, *aggR* positive, *aggA*(AAF/I) positive, ESBL resistance (Frank *et al*., 2011).

Growth curves were generated using the Bioscreen C device (Labsystems, Helsinki, Finland). 200 µl of culture containing 200 bacteria were inoculated into the wells of a 100- well plate. Growth was monitored at 37°C with moderate shaking (40 move instruction setting) by measuring optical densities at 600 nm wavelength in 20 min intervals until stationary phase was reached.

Generation of starvation microcosms
To generate microcosms, *E. coli* O104:H4 (11-02027) was grown overnight on chromID ESBL agar medium (bioMerieux, Germany) to ensure ESBL plasmid retention. Bacteria were then adjusted to 2 x 10^8 cfu ml^{-1} by inoculating bacteria into sterile 0.9% saline to an OD660 of 0.3. 40 ml filter-sterilized starvation solutions (tap water originating from town 1 (tap 1), tap water from town 2 (tap 2), 0.9% saline (PK), and saline containing 500 µM CuSO4) were inoculated with 200 µl of bacterial suspensions yielding a final bacterial density of 10^6 cfu ml^{-1} each. Tap 1 and 2 were chosen for the experiments with the *E. coli* O104:H4 2011 outbreak strain due to the radical differences in culturability kinetics of different *Salmonella enterica* strains, which has been previously observed (P. Aurass and A. Flieger, unpubl. obs.). The abiotic parameters of the two tap waters were determined according to the German Drinking Water Ordinance (Fig. S1). Since tap 1 but not tap 2 was disinfected with the

addition of chlorine dioxide (0.2 mg l^{-1}) at the water purification plant before delivery to the households, it is possible that tap 1 contains trace amounts of hypochlorite and hypochlorous acid (personal communication with the water purification plants). Bacterial cfu in the inoculum were determined by plating of serially diluted samples. Each experimental condition was set up in duplicates in 50 ml plastic tubes. Duplicate samples of each starvation condition were incubated at 23°C or 4°C respectively.

To determine the state of culturability of suspensions, 10 µl aliquots were spot plated onto NA plates and grown for 1 day until culturable counts dropped below detection. Subsequently, 1 ml aliquots were plated until cfu dropped below $1/10^6$, and cultures with less than one cfu in 10^6 total bacteria are referred to as zero cfu state cultures.

Determination of live/dead state and microscopy

For live/dead discrimination 1 ml culture aliquots were removed and stained with the propidium iodide (Pi)/Syto9 dyes, both included in the Live/Dead BacLight Kit (Invitrogen L7007). For this purpose, 0.5 µl solution A (containing 1.67 µM Pi + 1.67 µM Syto9) and 1 µl of solution B (containing 18.3 µM Pi + 1.67 µM Syto9) were added directly to the culture aliquot and were incubated for 15 min in the dark. Subsequently, stained suspensions were filtered through black nucleopore filters (Pall) and imaged at 400- or 1000- fold magnification in a Nikon Eclipse fluorescence microscope. Individually stained cells were either automatically counted using the CellC software (Selinummi *et al*., 2005) or counted manually for control purposes.

Brightfield microscopy

DIC images were generated using a Nikon Eclipse microscope equipped with nomarsky optics. Bacteria were imaged on agarose coated glass slides with 1000-fold magnification.

Resuscitation of copper ion-stressed bacteria

One millilitre aliquots of microcosms containing copper ionstressed VBNC bacteria generated at 4°C were treated with 1/1000 volume of 500 µM EDTA and incubated 3 min on ice. Subsequently, bacteria were pelleted by centrifugation in a refrigerated centrifuge (5 min 7000 *g*). Supernatants were removed, and bacteria were resuspended in 1 ml cold 500 µM EDTA in 0.9% saline solution. The wash step was repeated once more, and bacteria were finally resuspended to the original volume in 0.9% saline. 100 µl aliquots (theroretically containing 10^5 bacteria) were spot plated on NA or NA containing 2000 U catalase (Sigma-Aldrich). In parallel, controls containing non-EDTA washed aliquots of the same respective unculturable microcosm were plated in 100 µl spots and 1 ml patches onto NA and NA containing 2000 U catalase to confirm the inability of the included bacteria to form colonies. Control plates and plates containing EDTAwashed bacteria were incubated at 25°C for 7 days and checked daily for cfu. After 3 days, no further increase in cfu formation was observed in all subsequent experiments.

Multiplex PCR of resuscitated bacteria for confirmation of E. coli *O104:H4 characteristics*

Multiplex PCR was carried out using the primer pairs LP43/LP44, TerD1/TerD2, 104rbfO-f/104rbf-r and fliCH4-a/fliCH4-b as described elsewhere (Bielaszewska *et al*., 2011). PCR was carried out using a thermocycler 'Flexcycler' (analyticjena, Jena, Germany) and *Taq* DNA polymerase (New England Biolabs, Frankfurt am Main, Germany).

PCR for confirmation of pAA virulence gene loci

The *aggA* (AAF/I) PCR was performed according to (Tsai *et al*., 2003). The PCR for the *aggR* gene was carried out according to Czeczulin and colleagues (1999).

Characterization of plasmids and PFGE analysis

Large plasmids were isolated and plasmid profiles were analysed by agarose gel electrophoresis according to Prager and colleagues 2005 (Prager *et al*., 2005). PFGE was performed following the PulseNet protocol of the CDC, Atlanta (Hunter *et al*., 2005). The PFGE Patterns were analysed with BioNumerics, Version 5.1 (Applied Maths, BVBA, Belgium).

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Tables and Figures

Table 1. Persistence and non-culturability of *E. coli* O104:H4 2011 outbreak strain (11-02027) in different environments.

	cfu ml^{-1} at day 0	0 cfu state at day	cfu ml^{-1} at day 10	cfu ml^{-1} at day 40
4°C				
PK	9×10^5	–	n.d.	5×10^5
PK + Cu^{2+}		5	0	0
Tap 1		–	7×10^3	2×10^1
Tap 2		–	n.d.	3×10^5
23°C				
PK	9×10^5	–	n.d.	3×10^5
PK + Cu^{2+}		3	0	0
Tap 1		3	0	0
Tap 2		–	n.d.	2×10^4

n.d., not determined; results are representative for two additional independent experiments.

Figure 1. Copper ion- or tap 1-stressed, non-culturable *E. coli* O104:H4 populations contain viable bacteria. A. 10 days incubated *E. coli* O104:H4 (11-02027) populations in 0.9% saline (culturable control) or 0.9% saline + 500 µM Cu^{2+} (did not contain culturable bacteria) both at 4°C, and 40 days incubated Cu^{2+}-stressed, unculturable populations at 4°C and 23°C were double stained with propidium iodide (Pi) and Syto9 to determine bacterial viability. Images were taken at 1000-fold magnification by means of fluorescence microscopy. Boxes show magnified bacterial aggregates with membrane-intact living (green = Pi-/Syto9+) and membrane-damaged apparently dead (red = Pi+/Syto9-) cells found in all microcosms. B. Incubations for 40 days at 4°C (low fraction ≤ 0.01% still culturable) or 23°C (non-culturable) in tap 1 still contain living bacteria. C. Mean percentages of living and dead bacteria within the incubated cultures were determined. Increased temperature significantly reduced the proportion of viable cells from 56% at 4°C to 5% at 23°C (Cu^{2+} stress) or from 77% at 4°C to 17% at 23°C (tap 1 stress) respectively. The results represent the means of at least 500 counted bacteria per sample ($P \leq 0.01$ for fractions of dead cells; Student's *t*-test). Experiments were performed in duplicate cultures and are representative for two additionally performed independent experiments.

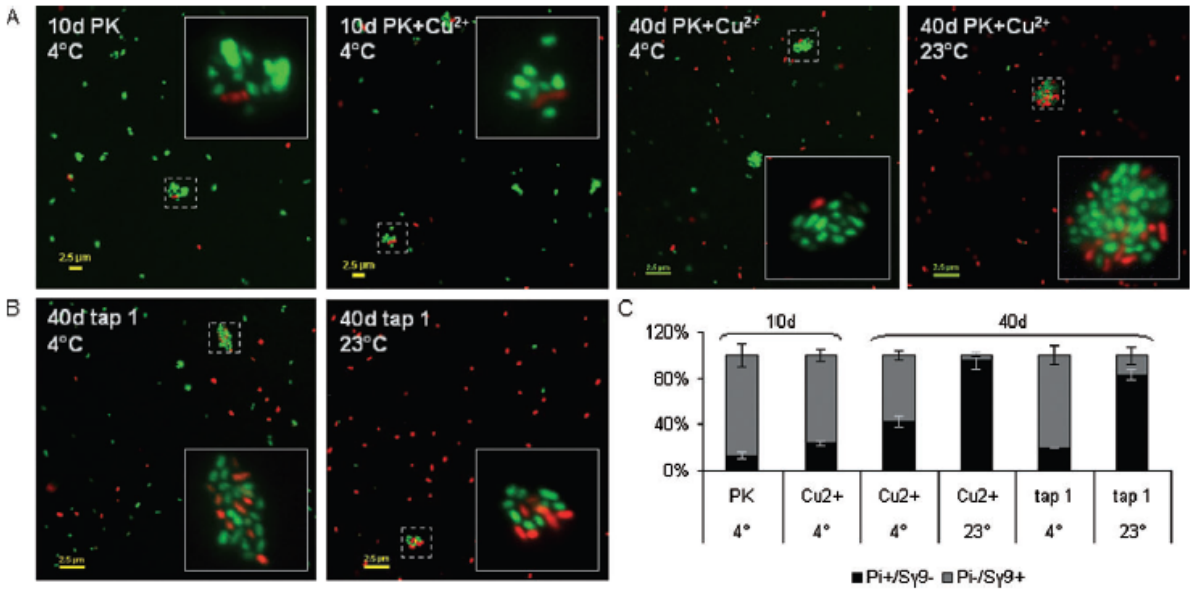

Figure 2. Resuscitation of copper ion-induced VBNC cells and conservation of strain characteristics. Within the 6 and 11 days (corresponding to 1 and 6 days after 0 cfu state respectively) Cu^{2+} treated non-culturable *E. coli* O104:H4 (11-02027) population at 4°C, colony-forming ability was restored for a fraction of bacteria by repetitively washing the bacteria with cold 500 µM EDTA in 0.9% saline and subsequent plating onto rich medium (NA) supplemented with 2000 U catalase per plate (A). To confirm the identity with the parental strain and preservation of strain characteristics after resuscitation, resuscitated clones were analysed for: strain characteristic and virulence loci (1 = *stx2*, 2 = *terD*, 3 = *rbfO104*, 4 = *fliC H4*) by PCR (B), genetic similarity via XbaI macrorestriction and PFGE (C), plasmid profile (showing the 90 kbp antibiotic resistency plasmid and the 83 kbp pAA EAEC virulence plasmid (Mellmann *et al*., 2011) (D), retention of the pAA virulence plasmid by PCR amplification of *aggR* and *aggA* (E), and comparable growth kinetics starting growth from a low inoculum (1000 cfu ml^{-1}) in LB broth (F). Abbreviations: parental strain (+), three representative resuscitated clones (rs1, 2, 3), PCR negative control containing water (-).

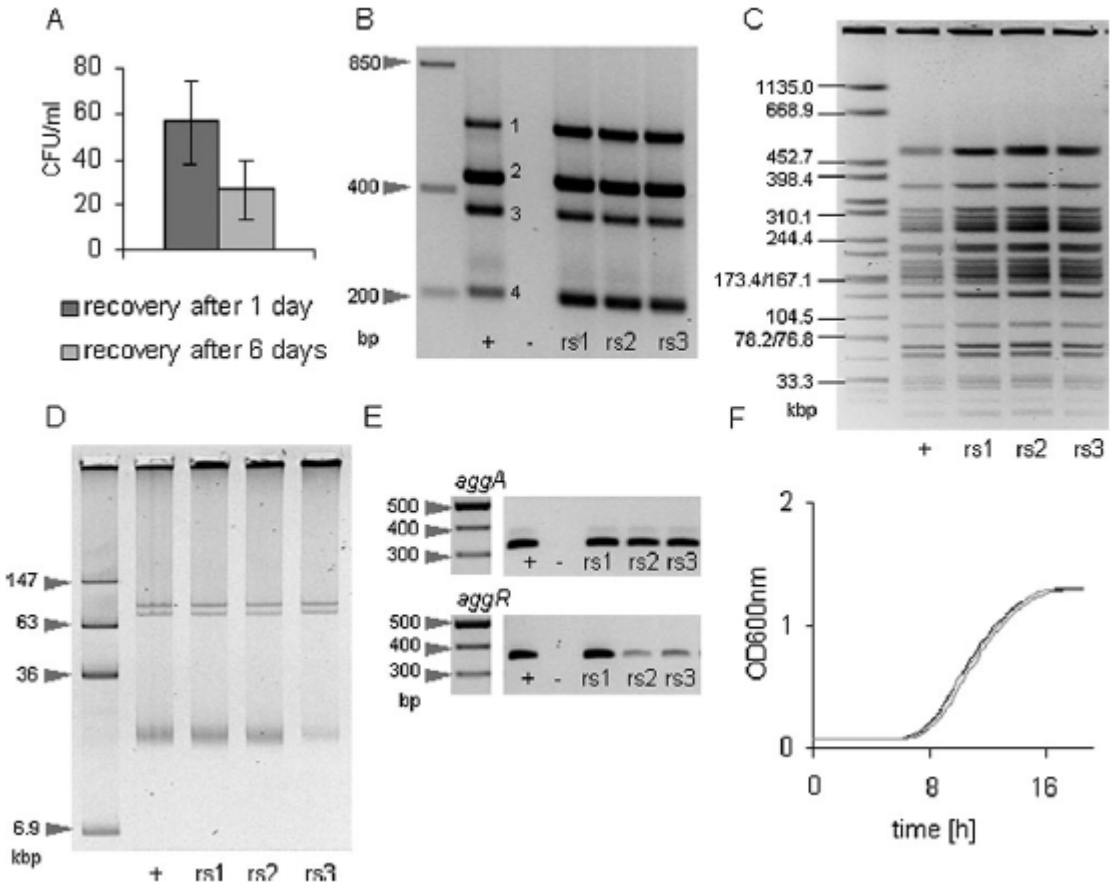

Figure 3. The broth-grown parental *E. coli* O104:H4 2011 outbreak strain and the resuscitated strain show characteristic autoaggregation. DIC images of parental (A), of 10 day copper ion-stressed VBNC (B), and resuscitated (C) *E. coli* O104:H4 2011 outbreak strain clones are shown. Strains were grown overnight in LB broth to stationary phase and were then imaged at 1000-fold magnification. DIC image (1000-fold magnification) of the 10 days at 4°C copper ion-stressed population containing VBNC cells (VBNC) shows dwarfing of bacteria compared with the resuscitated (culturable) population after enrichment in nutrient-rich broth (B, C and D). The results represent the means and standard deviations of at least 200 measured cells per sample (P <0.01 for diameter and length; Student's t-test).