Microbiology Focus

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Fluka Analytical

Clostridium perfringens — an indicator

Surface water a source of *Clostridium perfringens*
(source *C. perfringens* FISH image: BioVisible BV, The Netherlands)

Clostridium perfringes — an Indicator

By Prof. Mohammad Manafi, Medical University of Vienna
mohammad.manafi@meduniwien.ac.at
Jvo Siegrist, Sigma-Aldrich
ivo.siegrist@sial.com

Clostridium perfringens is an effective indicator of faecal contamination. Unlike *E. coli, C. perfringens* can withstand processing treatments and unfavorable growth conditions.

Clostridium perfringens is found in undercooked or improperly sterilized canned foods (germination of endospores) and in water (surface water). The natural contamination source is human and animal faeces transmitted into food products primarily by water. *C. perfringens* produces an extensive range of invasins and exotoxins. The enterotoxins cause the undesirable, predominantly meat-associated, food poisoning, as well as wound and surgical infections that lead to gas gangrene.

C. perfringens plays a subsidiary role in water examination [6]. *Clostridia* are spore builders and are resistant to heating, chlorination and other stress factors. In contrast to vegetative cells like coliforms *(E. coli,* enterococci*)*, which are less resistant, *C. perfringens* has the advantage of a robust capability to survive [8]. Therefore, while faecal contamination is detected primarily by coliforms as an indicator, which could disappear after a processing step, *C. perfringens* remains present. The organism is not a hazard in water; rather, it is problematic when the water comes in contact with food.

In consideration of the aforementioned facts, it is obvious that detection and identification of *C. perfringens* is an important step toward the control and eradication of this potent pathogen. *C. perfringens* is an anaerobic, Gram-positive, spore-forming rod-shaped **(see Figure 7, pg 4)** bacterium [3, 5]. Some characteristic enzymes of *C. perfringens* are hemolysins (β-hemolysis), lecithinase, extracellular proteases, lipases (phospholipase-C), phosphatase, collagenase, hyaluronidase, saccharolytic enzymes and enzymes to reduce sulphite to sulphide. These enzymes are also used as detection and differentiation targets. It is also notable that *C. perfringens* is a non-motile bacterium, and it is the most important of the sulphite reducing clostridia [5].

Also, *C. perfringens* normally grows at 44 °C, whereas some other clostridia are inhibited at this temperature. This property is used in ISO methods to give the medium more selectivity [4].

Early detection of *Clostridium* in food is important to control outbreaks. To facilitate detection, Fluka has introduced a new chromogenic media, CP *ChromoSelect* Agar, for enumeration and differentiation of *Clostridium sp.*, in particular *Clostridium perfringens*, in aqueous samples **(Figure 1)**.

For the standard detection of *C. perfringens*, mCP and TSC agar have been recommended [6, 7]. However, there are problems associated with each of these media. CP *ChromoSelect* Agar is more reliable and easier to handle than m-CP and TSC agars. The color does not diffuse in the agar and confirmation is not required since the green coloration is specific for *C. perfringens* [9]. CP *ChromoSelect* Agar avoids the disadvantages of m-CP agar, including the presence of ammonia, which prevents subculturing the *C. perfringens* colonies, the overly-selective nature of m-CP agar, and the evanescence of the red color of colonies after the addition of ammonia, which makes further confirmation impossible. In addition, the recovery of *C. perfringens* was rejected by ISO in favor of methods based on TSC agar [1, 8]. CP *ChromoSelect* Agar also eliminates the excessive and variable blackening of the peripheral colonies encountered with TSC agar, which makes colony counting at lower dilutions difficult and leads to false positives **(Figure 2)**. It is also more reliable at high bacteria counts, where the TSC agar can produce false negatives because of an interference with the other enzymatic mechanisms from acid production and oxygen contact **(Figure 3)**. TSC detects all

Figure 1: Drinking water sample cultured on CP *ChromoSelect* Agar. *C. perfringens* appears as distinct green colonies.

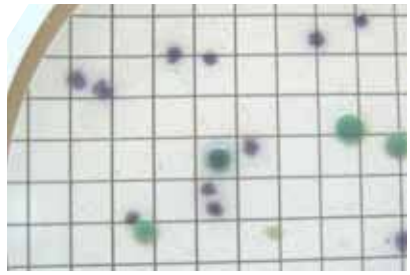


Figure 2: Comparison of m-CP, TSC and CP ChromoSelect Agar (from left to right)

Figure 3: *C. perfringens* ATCC 10873 on CP *ChromoSelect* Agar (above) and TSC agar (below). Note the false negatives on the TSC agar.

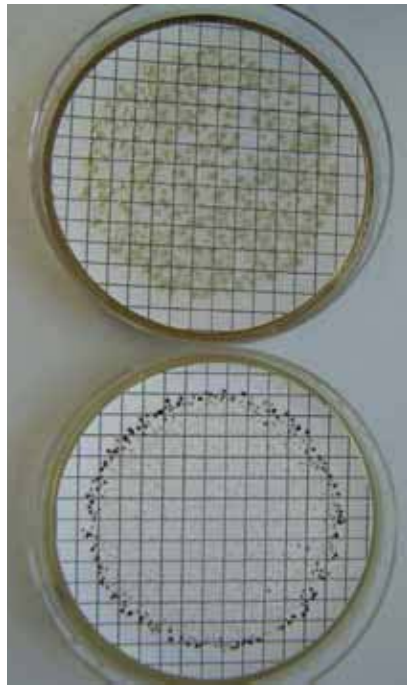


Figure 4: TSC agar + fluorogenic substrate = TSCF agar

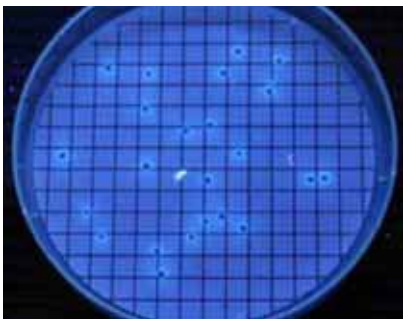
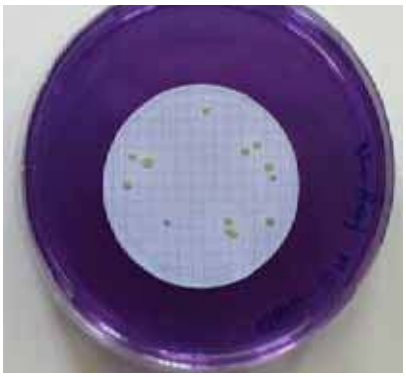
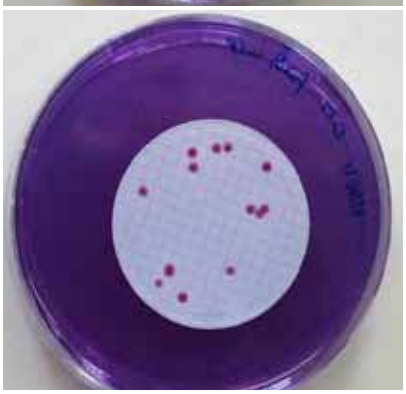


Figure 5: *C. perfringens* on mCP Agar before (above) and after (below) ammonia hydroxide vapors





sulphite-reducing clostridia, however, and not only *C. perfringens*. TSC agar can be improved with the addition of MUP, a fluorogenic substrate which helps to confirm the presence of phosphatase. This modification is designated as TSCF **(Figure 4)**.

In the present study, three media types (mCP **[Figure 5]**, TSCF and CP *ChromoSelect* Agar) were evaluated for recovery of *C. perfringens* in various surface water samples. Using a membrane filtration technique on 139 water samples, 131 samples (94.2%) were found to be presumably positive for *C. perfringens* in at least one of the culture media. Green colored colonies on CP *ChromoSelect* agar **(Figure 1)** were counted as presumably *C. perfringens* isolates.

Out of 483 green colonies on CP *ChromoSelect* Agar, 96.9 % (465 strains, indole negative) were identified as *C. perfringens*, 15 strains (3.1%) were indole positive and were identified as *C. sordelli, C. bifermentans* or *C. tetani*. Only 3 strains (0.6 %) gave false positive results and were identified as *C. fallax, C. botulinum,* and *C. tertium* **(Table 1)**. Variance analysis of the data obtained shows no statistically significant differences in the counts obtained between media employed in this work **(Figure 6 and Table 2)**.

Table 1: All green colonies isolated from CP *ChromoSelect* Agar and identified with API system (n=483)

strains	Indole reaction	n
C. perfringens	-	465 (96.3%)
C. tertium	-	1 (0.2%)
C. botulinum	-	1 (0.2%)
C. fallax	-	1 (0.2%)
C. bifermentans	+	2 (0.4%)
C. sordelli	+	12 (2.5%)
C. tetani	+	1 (0.2%)

Figure 6: Comparison between TSCF agar and the mCP and CPC (CP *ChromoSelect* Agar) media for enumerating strains of *C. perfringens* in water samples.

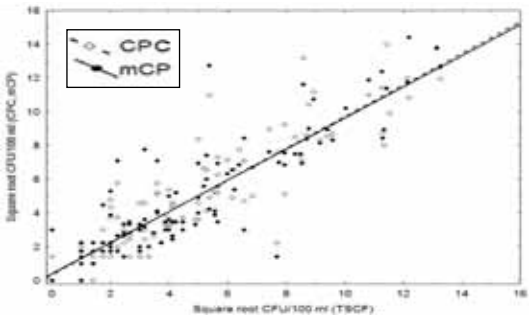


Table 2: Analysis of variance of *C. perfringens* spore counts obtained on all media

Effect	Sum of squares	df	Mean square	F ratio	p value
Media	0.392	2	0.196	0.190	0.827
Residual	235.578	228	1.033		

In general, the identification of typical and atypical colonies isolated from all media demonstrated that CP *ChromoSelect* Agar was the most useful medium for *C. perfringens* recovery in water samples [2].

In addition to its advantages over m-CP and TSC agars, CP *ChromoSelect* Agar is an ideal growth media. It contains only vegetable peptones and, together with yeast extract, it is an excellent source of nitrogen, carbon, amino acids and vitamin B complex. Sucrose acts as the fermentable carbohydrate and reducing agents lower the redox potential of the media. Diverse salts provide the required ions for enzymatic reactions. Buffering agents stabilize the pH within the ideal growth range. Inhibitors D-cycloserine and polymyxin B give the medium its selectivity, while further selectivity is achieved by incubation under anaerobic conditions at 44 °C. Various promoters and substrates protect injured cells to improve recovery rate and enhance growth. The chromogenic enzyme substrates in the CP *ChromoSelect* Agar provide the differentiation, particularly for *C. perfringens* **(Table 3)**. A negative indol reaction (Kovac's Reagent) confirms the presence of *C. perfringens*.

Table 3: *Clostridium sp.* cultural characteristics in CP *ChromoSelect* Agar

Organisms (ATCC)	Growth	Colony Appearance
Clostridium perfringens (13124)	+++	Green
Clostridium bifermentans (638)	+++*	Dark blue with violet halo
Clostridium sporogenes (8534)	-	-
Clostridium sordelli (9714)	++	Dark green with halo (change to red with Kovac's Reagent)
Enterococcus faecalis (29212)	++	Violet
Escherichia coli (25922)	-	-
Pseudomonas aeruginosa (27853)		Colorless
Staphylococcus aureus (25923)	-	-
Bacillus subtilis (6051)	-	-
Salmonella typhimurium (DSM 554)	++	Violet

* Growth at 40 °C, but no growth at 44 °C

Did you know...

Clostridium perfringens has one of the highest reproduction rates in the bacteria kingdom. Under optimal conditions, the cell doubles every 10 minutes!

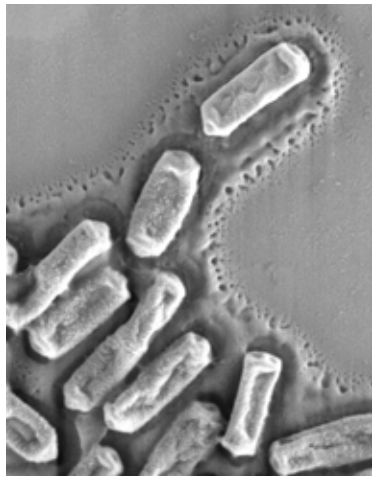


Figure 7: Scanning electron micrograph of *C. perfringens* grown on a silicon wafer (source: S. Melville, Department of Biological Sciences, Virginia Tech University)

Table 4: Typical Media for *Clostridium perfringens*

Nonselective Media	Cat. No.	Description
AEA Sporulation Broth (Base), modified *Supplements: 4 g/l raffinose (Fluka 83400) + 930 mg/l sodium carbonate (Fluka 71351) + 42.6 mg/l cobalt chloride (Sigma-Aldrich 60818) + 20 mg/l sodium ascorbate (Sigma 11140)*	17170	For early sporulation of *Clostridium perfringens* from foods.
Brewer thioglycollate medium	B2551	Used for testing the sterility of biological products.
Clostridial Nutrient Medium*	27546	For the cultivation and enumeration of clostridia and other anaerobes as well as facultative microorganisms in food, clinical and other material acc. to Hirsch & Grinsted.
Cooked Meat Broth	60865	For the primary cultivation of aerobic, microaerophilic and anaerobic bacteria from clinical specimens.
Reinforced Clostridial Agar	91365	Used for the cultivation and enumeration of Clostridia.
Nonselective Differential Media	**Cat. No.**	**Description**
Clostridial Differential Broth*	27544	For the MPN enumeration of all clostridia in food and other material acc. to Gibbs and Freame.
Gelatin Iron Agar	G0289	Used for detecting gelatin liquefaction and hydrogen sulphide production.
Lactose Gelatin Medium (Base)* *Supplement: 120 g/l gelatine* 180 Bloom (Fluka 48722)*	61348	For the detection of lactose and gelatine metabolizing microorganisms (Cl. perfringens).
Meat Liver Agar	46379	For the cultivation of anaerobic microorganisms.
Motility Nitrate Medium* *Supplement: 5 ml/l glycerol (Sigma 49767)*	14305	Selective medium for motile nitrate-utilizing microorganisms (*Ps. aeruginosa, Cl. perfringens*).
Nutrient Gelatin	70151	Nutrient Gelatin is recommended for the determination of gelatin-liquefying microorganisms as well as for the enumeration of proteolytic organisms in water by the plate count test.
Sulfite Iron Agar* *Supplement: 1.4 g/l ferrous sulfate (Fluka 44980)*	86128	For the detection and enumeration of *Clostridium* species in meat and meat products.

Table 4: Typical Media for Clostridium perfringens (Contd.)

Selective Differential Media	Cat. No.	Description
CP ChromoSelect Agar *Supplements: 2 vials/l M-CP selective Supplement I (Fluka 51962) or 2 vial/l of Perfringens T.S.C. Supplement (P9352)*	12398	A chromogenic Agar for enumeration detection and differentiation of *Clostridium sp.*, in particular *Clostridium perfringens*, in aqueous samples.
m-CP Agar Base *Supplements: 2 vial/l m-CP Selective Supplement I (Fluka 51962) + 2 vial/l m-CP Selective Supplement II (Fluka 82265)*	75605	Recommended by the Directive of the Council of the European Union 98/83/EC for isolation and enumeratin of *Clostridium perfringens* from water sample using membrane filtration technique.
Perfringens Agar Base *Supplements: 50 ml/l Egg Yolk Emulsion (Fluka 17148) + 2 vial/l of Perfringens S.F.P. Supplement (53436) or 2 vial/l of Perfringens T.S.C. Supplement (P9352)*	39727	Used for the presumptive identification and enumeration of *Clostridium perfringens* from food.
SPS Agar*	85627	Medium for detection, isolation and enumeration of *Clostridium perfringens* and *Clostridium botulinum* in food acc. to Angelotti et al. (1962).
SPS Agar, modified	17231	For the selective isolation and enumeration of *Clostridium perfringens* from foods.
TSC Agar* *Supplements: TSC Agar: 400 mg/l D-cycloserine (Sigma 30020) or 2 vial/l Perfringens T.S.C. Supplement (Fluka P9352) and 50 ml/l Egg Yolk Emulsion (Fluka 17148)* *TSC Agar egg yolk free: 400 mg/l D-cycloserine (Fluka 30020) or 2 vial/l Perfringens T.S.C. Supplement (Sigma/Fluka P9352)* *TSC Fluorescence Agar: 5.5 g/l rehydrated TSC Agar supplement (Fluka 80548) SFP Agar (Shahidi-Ferguson Perfringens Agar): 2 vial/l of Perfringens S.F.P. Supplement (Fluka P9477) and 50ml/l Egg Yolk Emulsion (Fluka 17148)*	93745	For the isolation and enumeration of vegetative forms as well as spores from *Clostridium perfringens* in food, clinical specimens and other material acc.to Harmon et al. (1971).
TSN Agar*	93735	Highly selective medium for the detection and enumeration of *Clostridium perfringens* in food and other material.

* not sold in USA

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The *Burkholderia cepacia* Complex: A Group of Multilingual Bacteria

By Leo Eberl, Department of Microbiology, Institute of Plant Biology, University Zürich
leberl@botinst.unizh.ch

In the world of microorganisms, some members of the genus *Burkholderia* are multilingual, utilizing at least three different communication systems.

Over the past 20 years, it has become evident that bacteria exist not only as individual cells; in addition, they often coordinate their activities and act in a concerted manner similar to that of multicellular organisms. Interactions between cells are brought about by cell-to-cell communication systems that depend on the production and detection of small diffusible signal molecules. This phenomenon, which has been termed 'quorum sensing' (QS) [1], provides bacteria with a regulatory mechanism to express certain phenotypic traits in a population density-dependent manner. To date, various signal molecules belonging to different chemical classes have been described.

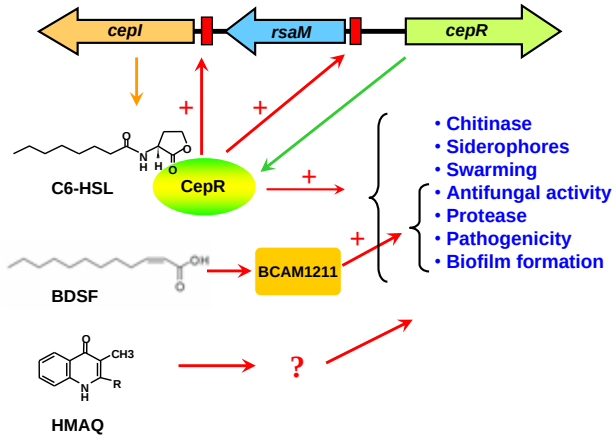
Some bacteria utilize more than just one communication system and therefore can be considered multilingual. One well-investigated example are the bacteria belonging to the *Burkholderia cepacia* complex (Bcc). The Bcc currently comprises a group of 17 formally-named bacterial species that, although closely related, are phenotypically diverse. Strains of the Bcc are ubiquitously distributed in nature and have been isolated from soil, water, the rhizosphere of plants, industrial settings, hospital environments, and from infected humans. Some Bcc strains have emerged as problematic opportunistic pathogens in patients with cystic fibrosis as well as in immunocompromised individuals [2].

All Bcc species investigated so far produce N-octanoylhomoserine lactone (C8-HSL), a member of the N-acyl homoserine lactone (AHL) signal molecule family, by the aid of the AHL synthase CepI. As the cell density increases, C8-HSL accumulates in the growth medium until a critical threshold concentration is attained. At this point, C8-HSL binds to its cognate LuxR-type receptor protein CepR, which, in turn, leads to the induction or repression of target genes **(Figure 1)**. Previous work has identified several QS-regulated functions in strains of the genus Burkholderia, including the production of extracellular proteases, chitinases, siderophores and the fungicide pyrollnitrin, swarming motility, biofilm formation and pathogenicity [3]. Moreover, some Bcc strains harbor additional AHL-based QS systems that utilize different AHL species to either fine-tune the CepIR QS system or regulate separate sets of genes.

In addition to AHLs, Bcc strains were demonstrated to produce diffusible signal factors (DSFs). While most strains synthesize cis-2-dodecenoic acid, which was named the *Burkholderia* diffusible signal factor (BDSF), some Bcc species also synthesize other DSF family molecules. Subsequent studies have shown that BDSF plays a role in the regulation of bacterial virulence and biofilm formation [4]. BDSF is synthesized by the gene product of Bcam0581 and appears to be sensed by the histidine sensor kinase BCAM0227. Interestingly, the AHL- and BDSF-dependent QS systems appear to act in conjunction in the regulation of *B. cenocepacia* virulence, as a set of the AHL-controlled virulence genes is also positively regulated by BDSF [4, 5]. Furthermore, mutation of Bcam0581 results in substantially retarded energy production and exhibits impaired growth in minimal medium, highlighting the dual roles of the BDSF QS system in physiology and infection.

Finally, Bcc strains were also shown to produce a third class of signal molecules, namely 4-hydroxy-2-alkylquinolines (HAQs). Recent work has shown that *Burkholderia ambifaria* (a member of the Bcc) HAQs contain predominantly an unsaturated aliphatic side chain and are typically methylated (at the 3 position), hence their designation as 4-hydroxy-3-methyl-2-alkylquinolines (HMAQs) [6]. Interestingly, inactivation of HAQs' production of *B. ambifaria* has led to an increased AHL production and, consequently, affected phenotypes that are under the control of QS in *B. ambifaria*, namely antifungal activity, siderophore production, and proteolytic activity.

Figure 1: Bcc strains can utilize up to three different cell-to-cell communication systems that rely on different classes of signal molecules: N-acyl-homoserine lactones (particularly C8-HSL), cis-2-dodecenoic acid, the Burkholderia diffusible signal factor (BDSF), and 4-hydroxy-3-methyl-2-alkylquinolines (HMAQs).



At present, it is unclear why Bcc strains synthesize multiple signal molecules. However, it should be kept in mind that these bacteria inhabit various habitats and thus may encounter a large diversity of bacteria. It is therefore tempting to speculate that the utilization of different communication systems will enable the bacteria to interact with a wide range of indigenous bacteria through chemical cross-talk. These interspecies interactions may be important for the fitness of the bacteria in their natural environment.

References

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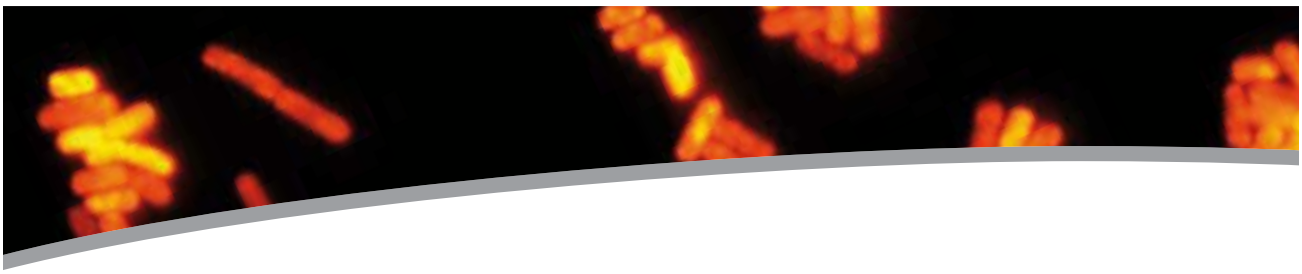
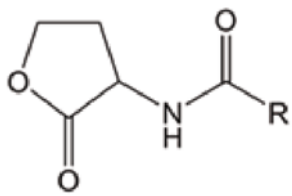


Sigma-Aldrich – Number One for Quorum Sensing Compounds



General chemical structure of an N-acyl homoserine lactone

Since 1998, Sigma-Aldrich has produced many Quorum Sensing Compounds and performed studies on their cell signaling reaction. The beginning efforts focused on simple N-acyl homoserine lactones (AHL) and an application test with the induction of violacein expression (violet pigment) in a *Chromobacterium violaceum* mutant (not able to produce AHL). Today the signaling molecules are emerging as more complex, while at the same time inhibiting compounds are growing in importance for research.

For more information, please visit our homepage: *sigma-aldrich.com/qs*

Quorum Sensing Substances

Cat. No.	Signalling Compounds	Substance
49619	BDSF	cis-2-Dodecenoic acid
10942	C4-HCT	N-Butyryl-DL-homocysteine thiolactone
09945	C4-HSL	N-Butyryl-DL-homoserine lactone
09926	C6-HSL	N-Hexanoyl-DL-homoserine lactone
10939	C7-HSL	N-Heptanoyl-DL-homoserine lactone
10940	C8-HSL	N-Octanoyl-DL-homoserine lactone
17248	C10-HSL	N-Decanoyl-DL-homoserine lactone
17247	C12-HSL	N-Dodecanoyl-DL-homoserine lactone
10937	C14-HSL	N-Tetradecanoyl-DL-homoserine lactone
42052	DSF	cis-11-Methyl-2-dodecenoic acid
53727	3-Hydroxy-C12-HSL	N-(3-Hydroxydodecanoyl)-DL-homoserine lactone
51481	3-Hydroxy-C14-HSL	N-(3-Hydroxytetradecanoyl)-DL-homoserine lactone
K3255	3-Oxo-C6-HSL	N-(3-Oxohexanoyl)-DL-homoserine lactone
K3007	3-Oxo-C6-HSL	N-(3-Oxohexanoyl)-DL-homoserine lactone
O1639	3-Oxo-C8-HSL	N-(3-Oxooctanoyl)-DL-homoserine lactone
O1764	3-Oxo-C8-HSL	N-(3-Oxooctanoyl)-L-homoserine lactone
O9014	3-Oxo-C10-HSL	N-(3-Oxodecanoyl)-L-homoserine lactone
O9139	3-Oxo-C12-HSL	N-(3-Oxododecanoyl)-L-homoserine lactone
O9264	3-Oxo-C14-HSL	N-(3-Oxotetradecanoyl)-L-homoserine lactone
07077	pC-HSL	N-(p-Coumaroyl)-L-homoserine lactone
94398	PQS (Pseudomonas quinolone signal)	2-Heptyl-3-hydroxy-4(1H)-quinolone
Cat. No.	**Antagonist/Inhibitors**	
53796	(Z-)-4-Bromo-5-(bromomethylene)-2(5H)-furanone (Furanone C-30)	
68749	3-Oxo-N-(2-oxocyclohexyl)dodecanamide	

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Tel: (+86) 21 6141 5566
Fax: (+86) 21 6141 5567

Czech Republic
Tel: (+420) 246 003 200
Fax: (+420) 246 003 291

Denmark
Tel: (+45) 43 56 59 00
Fax: (+45) 43 56 59 05

Finland
Tel: (+358) 9 350 9250
Fax: (+358) 9 350 92555

France
Free Tel: 0800 211 408
Free Fax: 0800 031 052
Tel: (+33) 474 82 28 88
Fax: (+33) 474 95 68 08

Germany
Free Tel: 0800 51 55 000
Free Fax: 0800 64 90 000
Tel: (+49) 89 6513 0
Fax: (+49) 89 6513 1160

Hungary
Ingyenes telefonszám: 06 80 355 355
Ingyenes fax szám: 06 80 344 344
Tel: (+36) 1 235 9063
Fax: (+36) 1 269 6470

India
Telephone
Bangalore: (+91) 80 6621 9400
New Delhi: (+91) 11 4358 8000
Mumbai: (+91) 22 2570 2364
Hyderabad: (+91) 40 4015 5488
Kolkata: (+91) 33 4013 8003
Fax
Bangalore: (+91) 80 6621 9550
New Delhi: (+91) 11 4358 8001
Mumbai: (+91) 22 4087 2364
Hyderabad: (+91) 40 4015 5488
Kolkata: (+91) 33 4013 8000

Ireland
Free Tel: 1800 200 888
Free Fax: 1800 600 222
Tel: (+353) 402 20370
Fax: (+ 353) 402 20375

Israel
Free Tel: 1 800 70 2222
Tel: (+972) 8 948 4100
Fax: (+972) 8 948 4200

Italy
Free Tel: 800 827 018
Tel: (+39) 02 3341 7310
Fax: (+39) 02 3801 0737

Japan
Tel: (+81) 3 5796 7300
Fax: (+81) 3 5796 7315

Korea
Free Tel: (+82) 80 023 7111
Free Fax: (+82) 80 023 8111
Tel: (+82) 31 329 9000
Fax: (+82) 31 329 9090

Malaysia
Tel: (+60) 3 5635 3321
Fax: (+60) 3 5635 4116

Mexico
Free Tel: 01 800 007 5300
Free Fax: 01 800 712 9920
Tel: (+52) 722 276 1600
Fax: (+52) 722 276 1601

The Netherlands
Free Tel: 0800 022 9088
Free Fax: 0800 022 9089
Tel: (+31) 78 620 5411
Fax: (+31) 78 620 5421

New Zealand
Free Tel: 0800 936 666
Free Fax: 0800 937 777
Tel: (+61) 2 9841 0555
Fax: (+61) 2 9841 0500

Norway
Tel: (+47) 23 17 60 00
Fax: (+47) 23 17 60 10

Poland
Tel: (+48) 61 829 01 00
Fax: (+48) 61 829 01 20

Portugal
Free Tel: 800 202 180
Free Fax: 800 202 178
Tel: (+351) 21 924 2555
Fax: (+351) 21 924 2610

Russia
Tel: (+7) 495 621 5828
Fax: (+7) 495 621 6037

Singapore
Tel: (+65) 6779 1200
Fax: (+65) 6779 1822

Slovakia
Tel: (+421) 255 571 562
Fax: (+421) 255 571 564

South Africa
Free Tel: 0800 1100 75
Free Fax: 0800 1100 79
Tel: (+27) 11 979 1188
Fax: (+27) 11 979 1119

Spain
Free Tel: 900 101 376
Free Fax: 900 102 028
Tel: (+34) 91 661 99 77
Fax: (+34) 91 661 96 42

Sweden
Tel: (+46) 8 742 4200
Fax: (+46) 8 742 4243

Switzerland
Free Tel: 0800 80 00 80
Free Fax: 0800 80 00 81
Tel: (+41) 81 755 2828
Fax: (+41) 81 755 2815

United Kingdom
Free Tel: 0800 717 181
Free Fax: 0800 378 785
Tel: (+44) 1747 833 000
Fax: (+44) 1747 833 313

United States
Toll-Free: 800 325 3010
Toll-Free Fax: 800 325 5052
Tel: (+1) 314 771 5765
Fax: (+1) 314 771 5757

Vietnam
Tel: (+84) 3516 2810
Fax: (+84) 6258 4238

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World Headquarters
3050 Spruce St.
St. Louis, MO 63103
(314) 771-5765
sigma-aldrich.com

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