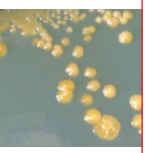
Microbiology Focus

Volume 4.3, 2012

Fluka Analytical

Cronobacter: The Infant Germ

Cronobacter species is a high risk for suckling babes. *Cronobacter* sp. on trypticase soy agar.



Cronobacter (Enterobacter sakazakii): The Infant Germ

By Jvo Siegrist, Product Manager Microbiology — *ivo.siegrist@sial.com*

Cronobacter is a problem in infant formula with the potential to cause a tragic outcome.

Cronobacter is a Gram negative, facultative anaerobic rod-shaped and motile bacterium and belongs to the Enterobacteriaceae family. It is closely related to the *Enterobacter* and *Citrobacter* species. *Cronobacter* was first described as yellow-pigmented *Enterobacter cloacae* (yellow pigment on a tryptic soy agar at 25 °C). However, there are also current studies that have demonstrated that only about 80% of *Cronobacter* spp. produce yellow-pigmented colonies on tryptic soy agar (see **Figure 2**). In the 1980's researchers used DNA-DNA hybridization to show that these strains were a unique taxonomic group and should be recognized as a separate species *'Enterobacter sakazakii'* (to honor the Japanese bacteriologist Riichi Sakazaki). The *Cronobacter* genus was defined first in 2007 and revised in 2008 based on studies of both partial 16S rDNA and hsp60 gene sequences, which showed that *'E. sakazakii'* isolates formed at least four distinct genomogroups which could be unique species. Today the genus is composed of *C. sakazakii, C. malonaticus, C.turicensis, C. muytjensii* and *C. dublinensis*, plus an unnamed sixth species. *Cronobacter* spp. can grow over a wide temperature range. It starts near refrigeration temperature (5.5 °C) and goes up to a growth temperature (44-47 °C), depending on the strain. The organism is very tolerant of drying steps and can survive for two years desiccated in infant formula and then rapidly grow on reconstitution.

Did you know…

Cronobacter starts to multiply at low temperatures?

Below 5.5 °C no growth was observed but at temperatures between 5.5 and 8 °C the multiplication begins. Average generation times of *Cronobacter* is about five hours at 10 °C and only 40 minutes at 23 °C.

Figure 1: Cell Division

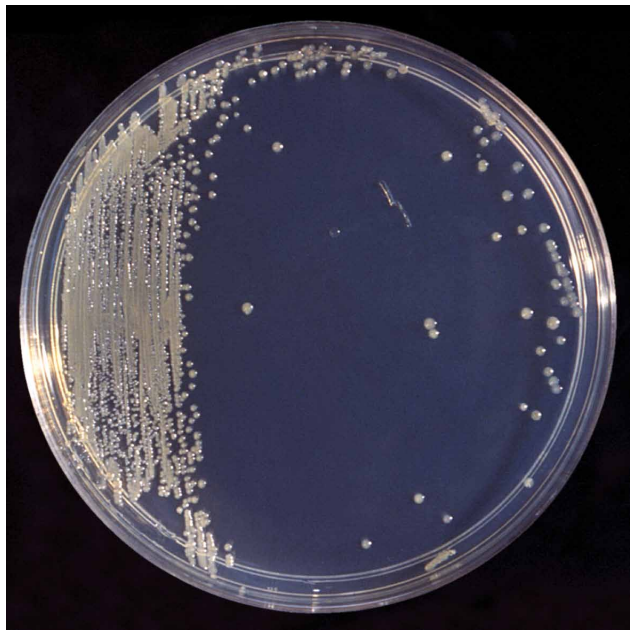


Figure 2: This inoculated trypticase soy agar (TSA) cultivated colonial growth of *Enterobacter sakazakii* bacteria that were slightly yellowish in color. (Source: CDC 1985)

Sources:

General *Cronobacter* is an ubiquitous organism that attaches to surfaces, forming biofilms that are resistant to cleaning and disinfectant agents. One probable natural source for *Cronobacter* is plant material, as it has been isolated from cereals, wheat, corn, soy, rice, herbs and spices, vegetables, and salads. The organism has also been isolated from a range of other foods including cheese, meat, milk powder, powdered infant formula and a large number of food ingredients.
The bacterium has been isolated from both the hospital environment and from clinical samples such as cerebrospinal fluid, blood, bone marrow, sputum, urine, inflamed appendix, neonatal enteral feeding tubes and conjunctivae. The *Cronobacter* species was also isolated from the stomach of flies. In the past, infant formula was the main problem, and there were many sad incidences of infection.

Clinical Facts:

The organism can cause severe neonatal infections: necrotizing enterocolitis, septicaemia and meningitis. The fatality rate following meningitis and other infections is 50%, with the survivors being neurologically damaged for life. Fortunately, infections are rare in infants but they can occur in all age groups, admittedly with less severe clinical outcomes. *Cronobacter* spp. has been shown to invade human intestinal cells, replicate in macrophages, and invade the blood-brain barrier.

Detection and Identification:

For the classic microbiological tests, a pre-enrichment step is used to recover the stressed cells, followed by a selective enrichment step.

ISO/TS 22964:2006 methods recommend buffered peptone water (BPW) as a pre-enrichment medium and modified lauryl sulphate broth with vancomycin (mLST) incubated at 44 °C for the secondary selective enrichment step. The next step is then a chromogenic agar for isolation and identification (see **Table 2**).

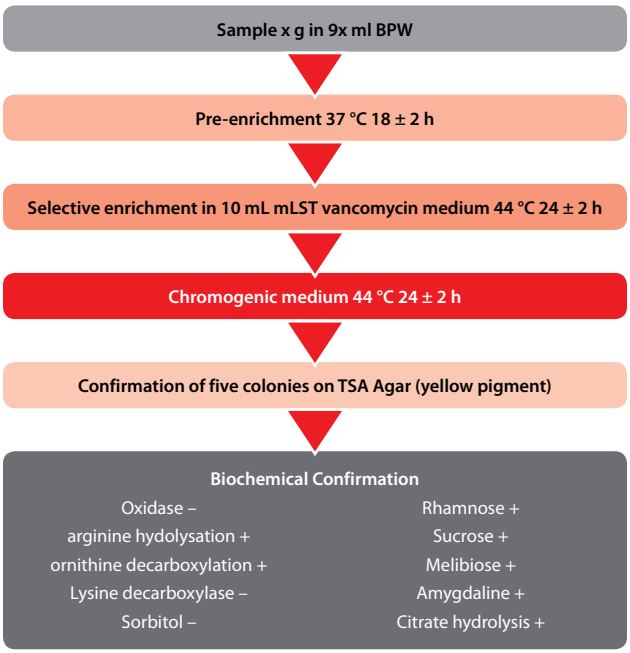


Figure 3: Method Flow Scheme ISO/TS 22964:2006

Since March 2012, the FDA method (see Bacteriological Analytical Manual) recommends Buffered Peptone Water (BPW) for enrichment (24 h, 36 °C), which is then centrifuged and resuspended in phosphate buffered saline followed by a PCR screening. The resuspended sample can also be streaked onto a chromogenic agar and typical colonies would then be confirmed by an oxidase test and other biochemical tests or an immunological method with PCR. **Table 1** lists the diverse biochemical reactions from *Cronobacter* species (not only from FDA).

There are diverse chromogenic agars available which help to save both time and effort in producing results and are more reliable than traditional media. The detection principle is based on the alpha-glucosidase possessed by *Cronobacter* spp. (not by most other Enterobacteriaceae) which cleaves the 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside or similar substrates. In the case of *Cronobacter* spp., the result is a plate with blue colonies (see **Figure 1**); however, phenotyping or genotyping confirmation is still required to avoid false positive due to organisms such as *E. vulneris* and *E. hermanii*.

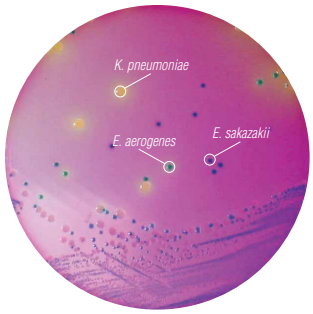


Figure 4: HiCrome™ *Cronobacter* spp. Agar (Fluka 92324); *Cronobacter* (blue), *E. aerogenes* (green), *K. pneumoniae* (yellow)

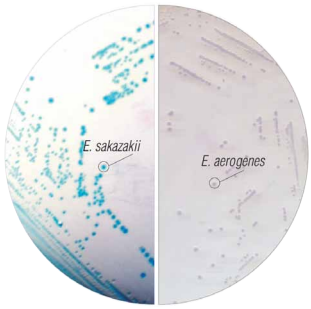
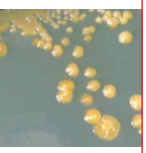


Figure 5: HiCrome *Cronobacter* spp. Agar, modified as recommended by ISO 22964:2006; *Cronobacter* (blue-green), *E. aerogenes* (colourless)

Biochemical Test	Cat. No. of Test	Reaction of *Cronobacter*
Gram	77730	–
oxidase	40560	–
catalase	88597	+
H_2S production	06728	–
nitrate reduction	51138	+
citrate utilization	85463	+
esculin hydrolyzation	06105	+
arginine hydolysation	D2935	+
Lysine	D2935	–
L-ornithine decarboxylation	D2935	+
Urease	51463	–
Indole	67309	–
ONPG	49940	+
D-adonitol	55876	–
L-arabinose	80372	+
D-arabitol	A3381	–
D-cellobiose	56481	+
Dulcitol	73044	–
D-fructose	53901	+
D-glucose	63367	+
D-galactose	89608	+
Inositol	89614	+ (75%)/–
Inulin	90058	+
Lactose	28816	+
Malonate	63290	+/–
D-maltose	77653	+
D-mannitol	94438	+
D-mannose	94445	+
D-melibiose	93196	+
x-methyl-D-glucoside	66940	+ (90%)/–
D-raffinose	94226	+
L-rhamnose	93999	+
Salicin	92971	+
Sorbitol	93998	–
D-sucrose	94309	+
D-trehalose	92961	+
Xylose	07411	+
Voges-Proskauer test (acetoin production)	07689	+
methyl red test	08714	–
tryptic soy agar at 25 °C	22091	yellow pigmented
Growth in KCN	60178	+

Table 1: Biochemical Reactions of *Cronobacter* spp.



Cat. No.	Description
Nonselective pre-enrichment	
77187	Peptone Water, phosphate-buffered (ISO/TS 22964)
Selective pre-enrichment	
89916	Modified Lauryl Sulfate Tryptose Broth (ISO/TS 22964) + Vancomycin supplement (Fluka 75423)
69965	Mossel Broth (FDA)
Isolation and differentiation	
92324	HiCrome™ *Cronobacter* spp. Agar*
14703	HiCrome *Cronobacter* spp. Agar, modified (ISO/ TS 22964)*
22091	Tryptic Soy Agar
79872	Tryptic Soy Agar (ready prepared in Flask)
70189	Violet Red Bile Glucose Agar

Table 2: Media for *Cronobacter* detection
* Not available in USA

Molecular Biological System:

The slow realization and recognition of *Cronobacter* as its own genus reflects the laborious, time consuming methods used in this pre-genomic period for bacterial characterization.

Therefore, Sigma-Aldrich provides a rapid, qualitative and quantitative detection of *Cronobacter* species by means of nucleic acid (rRNA) based methods in food such as infant formula.

- Time saving
- Easy handling
- Analysis in 2.5 hours
- Only living cells are detected (rRNA quickly decomposes in a dead cell)
- Sensitive (up to 1 CFU/mL with enrichment step)
- High specificity of the probes (low cross reaction)
- Robust system, not sensitive sample matrix
- Cost-effective analyses (96-well microplate format)

Principle of the Method:

The HybriScan™ method is based on the detection of rRNA via hybridization events and specific capture and detection probes. Sandwich hybridization is very sensitive, detecting attomoles of the respective target rRNA molecules. The ideal hybridization target for *Cronobacter* is rRNA, as the cells contain a large number of rRNA-containing ribosomes; a single cell therefore contains several thousand copies of rRNA but only one DNA. Sandwich hybridization also provides sensitivity in crude biological samples because it is not susceptible to matrix interference like PCR.

Specificity is achieved by targeting conserved or unique rRNA sequences. A biotin-labeled capture probe is used to immobilize the target sequence on a solid support plate (streptavidin-coated microtiter plate). A digoxigenin-labeled detection probe provides an enzyme-linked optical signal readout. Detection results from application of anti-DIG-horseradish peroxidase Fab fragments. The bound complex is visualized by horseradish peroxidase substrate TMB (3,3',5,5'-tetramethylbenzidine). Photometric data are measured at 450 nm and compared with standard solutions. The software enables easy measurement and data analysis.

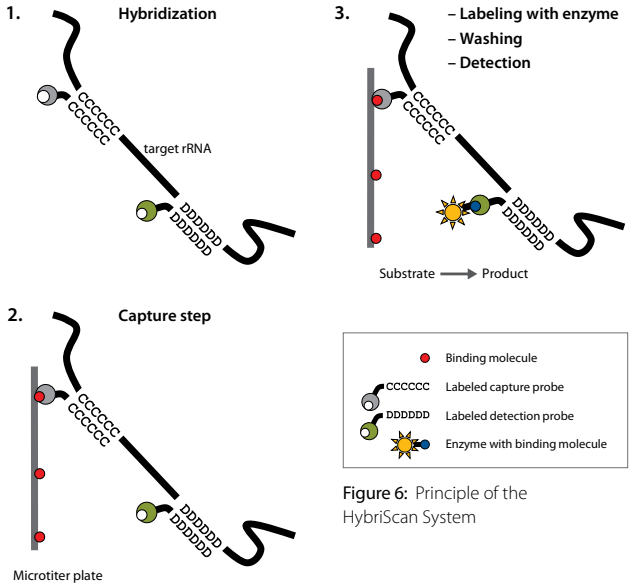


Figure 6: Principle of the HybriScan System

Figure 7 shows the specificity of HybriScan™***D*** *Cronobacter* spp. Different cell amounts and related Enterobacteriaceae were tested within a validation study. No signals were observed using 2,3 x 10^8 *Enterobacter cloacae* cells or 7 x 10^8 *Citrobacter freundii* cells per assay, whereas clear specific signals were detectable using 2,6 x 10^3, 1,3 x 10^4, and 2,6 x 10^4 cells of *Cronobacter* species, respectively. These results demonstrate that the HybriScan system is highly specific for *Cronobacter* spp.

The above mentioned data came out of a validation study from HybriScan™***D*** *Cronobacter* spp., which was performed using two different enrichment procedures: (1) single step enrichment for 24 – 26 hours at 37 °C in ESSB broth (*Enterobacter sakazakii* selective broth) and (2) two step enrichment starting with a pre-enrichment for 18 – 20 hours at 37 °C in buffered peptone water and followed by a selective enrichment for 24 – 26 hours at 45 °C in mLST selective broth.

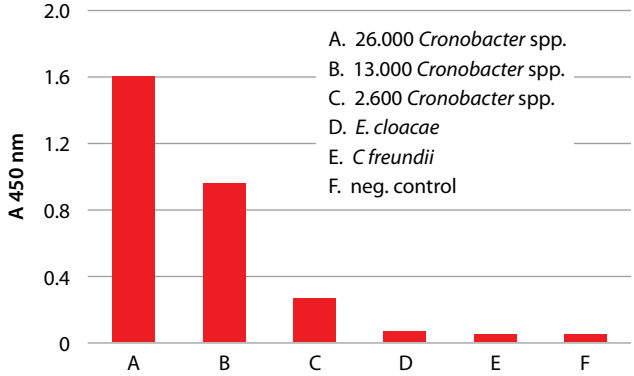


Figure 7: Specificity of HybriScan™***D*** *Cronobacter* spp. Different cell numbers of *Cronobacter* spp. and related Enterobacteriaceae like *E. cloacae* and *Citrobacter freundii* were tested. Measurement data for HybriScan analyses represent absorption at 450 nm.

Cat. No.	Description
12838-96TESTS	HybriScan™***D*** *Cronobacter* spp. Kit

An Interesting Chromogenic Medium for *E. coli* and Coliforms

By Jvo Siegrist, Product Manager Microbiology — *ivo.siegrist@sial.com*

A chromogenic medium for the differentiation and enumeration of *Escherichia coli* and other coliforms simplifies the membrane filtration technique for *E. coli* and coliforms.

The Membrane Lactose Glucuronide Agar (MLGA) reduces the number of filtration stages required from two to one and reduces the need for further confirmation steps. This medium is described in the Environment Agency's report of UK, "Methods for Examination of Waters and Associated Material – The Microbiology of Drinking Water (2002) – Part 4 – Methods for the isolation and enumeration of coliform bacteria and *Escherichia coli* (including *E. coli* O157:H7)". Basically it is a further development of the Membrane Lauryl Sulfate Agar by improving the recovery rate and the direct confirmation of the presence of β-glucuronidase.

For the analysis of treated liquids, 100 mL should be filtered through a filter (Φ 47 mm, pore size 0.45 µm) to get a reasonable number of colonies (20-80 colonies). In cases where a high number of bacteria is expected in the liquid, a smaller volume or a dilution should be filtered. The membrane filter is placed on the MLGA plate, making sure that no air bubbles are trapped between the membrane filter and the medium. The recommended incubation procedure is first 30 °C for four hours for the high recovery rate and then for 14 hours at 37 °C. Although an early indication is possible after 12 hours, the plates should be incubated for at least 18 hours to get a reliable result. The results should be read within 15 minutes after removing from the incubator as the yellow coloration may change on cooling and standing. *E. coli* gives green colonies while other coliforms are yellow. The green and yellow colonies are counted, then the amounts per milliliter are calculated and expressed in cfu/mL (colony forming units per mL).

Principle of the Medium System:

The chromogenic medium contains peptone and yeast extract, which provide amino acids, vitamins and other complex substances. Sodium lauryl sulphate inhibits gram-positive organisms. Sodium pyruvate protects injured cells, helps recovery of coliforms, and enhances growth. Lactose is a carbohydrate source which can be fermented by the coliform bacteria, using β-galactosidase, and the pH-indicator phenol red indicates this by changing to a yellow color (yellow colonies). X-Glucuronide is a chromogen substrate which can be cleaved by β-glucuronidase present in the *E. coli*. This results in a blue colony, but in combination with the lactose fermentation, it ends with a green colony. Normally, no further confirmation step is needed as the specificity of the green colonies being *E. coli* is very high. However, if a further confirmation step is preferred, there would be plenty of other tests like the Indole test, etc.

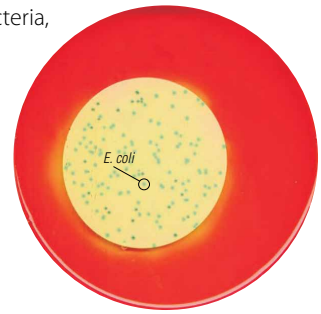


Figure 2: *E. coli* on MLGA

Blue colonies may be lactose-negative *E. coli*, but are more commonly strains of Aeromonas. Blue colonies should be classified as presumptive coliform bacteria and further testing is needed. It is seldom that yellow colonies confirm as *E. coli* (as some strains do not express β-glucuronidase or appear negative when first isolated) and green colonies may not confirm as *E. coli* but may, nevertheless, confirm as coliform bacteria. Some rare species of *Bacillus* and *Staphylococcus* may grow on membrane lactose glucuronide agar producing yellow colonies. They can be easily identified by colony characteristics on MacConkey Agar, and by Gram staining.

Cat. No.	Description
39734-500G-F 39734-88G-F	Membrane Lactose Glucuronide Agar Medium

Did you know…

Since 2009, the search term "chromogenic media", has often been searched for on Google.

If you look at the Google statistics, you can find the first peaks in 2007 and 2008 for this topic, and since 2009, it has become a regular search term. Sigma-Aldrich has developed and added new chromogenic media to its microbiology products for 15 years. The range of products has grown to three chromogenic broths and 33 chromogenic agars.

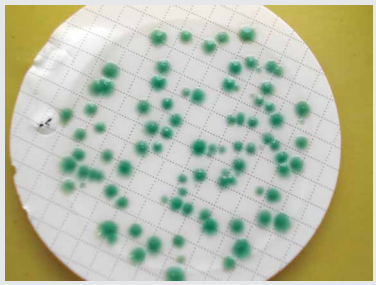
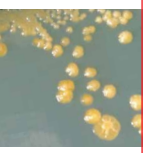


Figure 1: CP *ChromoSelect* Agar (Fluka 12398, *Cl. perfringens* as green colonies)



Vitroids™ – the Microorganism Standard

Reference microorganisms in certified and defined colony forming units (cfu)

- **Standards** in concentrations of 30–50'000 cfu per disc
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- **Certified** acc. ISO 17025
- **Delivered** with detailed certificate of origin
- **Reference** strains from ATCC, NCTC, etc.
- **Minimum** one year shelf life at 4 °C (usual two years)
- **No** lag-phase
- **Amazingly** little standard deviation (e.g. 100 cfu +/- 3%)

Cat. No.	Description
RQC01657	*Enterobacter aerogenes* ATCC® 13048 Vitroids 1000 CFU
RQC01655	*Enterobacter aerogenes* ATCC 13048 Vitroids 200 CFU
RQC01652	*Enterobacter aerogenes* ATCC 13048 Vitroids 50 CFU
RQC21102	*Enterococcus cloacae* ATCC 35030 Vitroids 50 CFU
RQC01777	*Enterococcus faecalis* ATCC 19433 Vitroids 1000 CFU
RQC01774	*Enterococcus faecalis* ATCC 19433 Vitroids 200 CFU
RQC01772	*Enterococcus faecalis* ATCC 19433 Vitroids 50 CFU
RQC01775	*Enterococcus faecalis* ATCC 19433 Vitroids 500 CFU
RQC01707	*Escherichia coli* ATCC 11775 Vitroids 1000 CFU
RQC01705	*Escherichia coli* ATCC 11775 Vitroids 200 CFU
RQC01702	*Escherichia coli* ATCC 11775 Vitroids 50 CFU
RQC11003	*Escherichia coli* ATCC 8739 Vitroids 80 CFU
RQC02504	Heterotrophic Organisms Vitroids 100 CFU

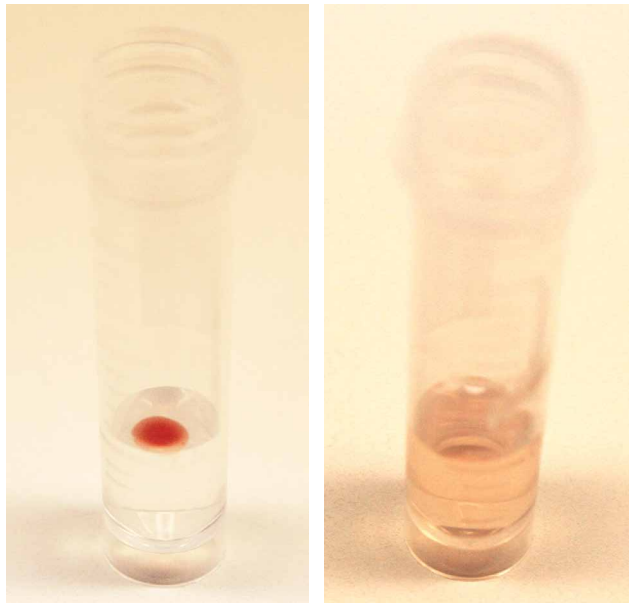
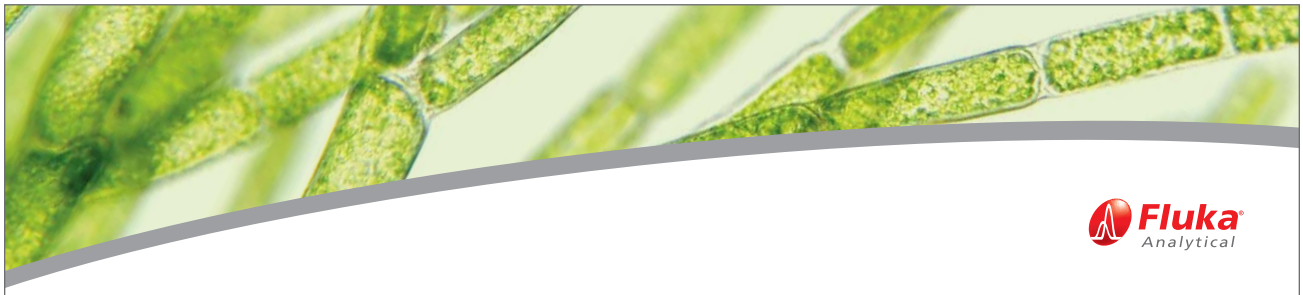


Figure 1: A Vitroid just put into buffer (left) and after 10 minutes it is completely dissolved (right)

More information and the full organisms list is found under
sigma-aldrich.com/vitroids



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Agar Description	Cat No.
For Baird Parker Agar	91411-500G
For membrane filtration, low gel strength	42146-500G
High purity, low ionic content, low gel strength	56763-500G
For chromogenic media	05729-500G
Anti-swarming	50524-500G

Some New Bifidus Media

By Jvo Siegrist, Product Manager Microbiology — *ivo.siegrist@sial.com*

Since 2010, the ISO and IDF have recommended a new selective TOS-propionate agar for the enumeration of Bifidobacteria. A few years ago Sigma-Aldrich developed a selective medium with an additional color reaction for confirmation.

Did you know…

that our intestinal flora started mainly with Bifidobacteria?

In adult intestines, only 3-6% of the fecal flora is composed of Bifidobacteria, while in breast-fed infants, Bifidobacteria can constitute up to 90%. With increasing age, the number of Bifidobacteria decreases. It was observed that babies and adults with lower numbers of Bifidobacteria have a higher risk for diarrhea and allergies.

Figure 1: Baby Drinking Infant Formula

Today Bifidobacteria are often used as the preferred probiotics. In most cases, they are produced directly by fermentation in foods such as yogurt, or are supplied through dietary supplements. Bifidobacteria are able to cleave prebiotics, which are non-digestible compounds, so prepiotics stimulate the growth and/or activity of probiotics, like *Bifidobacterium*, in the gut. Prebiotics are frequently used in functional food, and typically consist of oligosaccharides. Prebiotics can be found in milk (galactooligosaccharides or GOS) and in plants with dietary fibers, however they are often produced by fermentation.

Bifidobacterium is a Gram-positive, non-motile, rod-shaped, and often branched anaerobic bacteria. They were first isolated from a breast-fed infant by Henry Tissier from the Pasteur Institute. Diverse studies report the benefits of Bifidobacteria including a positive affect on the immune system, help to control intestinal pH, aid in the management or prevention of chronic intestinal inflammatory diseases, or of atopic diseases. In addition, bifidobacteria produce bacteriocins and bacteriocin-like inhibitory compounds which inhibit the growth of other pathogen bacteria. Potential beneficial applications abound, and the research on this topic continues to expand.

Bifidobacteria posess many glycosylases that are able to degrade various plant or milk-derived oligosaccharides. Several such enzymes were identified on the *Bifidobacterium* genome. Diverse glycosyl hydrolase, ABC transporter, and the FOS gene cluster that is involved in the processing of health-promoting fructooligosaccharides (prepiotics), called bifidogenic factors, can also be found on the genome. Obviously Bifidobacteria are able to utilize a broad range of substrates as energy sources, such as plant polymers, glycoproteins and glycoconjugates, as well as having specialized proteins for the catabolism of oligosaccharides.

Bifidobacteria also have a unique hexose metabolism called the bifid shunt. The key enzyme, fructose-6-phosphate phosphoketolase, is not found in any other Gram-positive intestinal bacteria.

Bifidobacteria are added as a probiotic supplement to infant formulas, drinks, yogurts and a range of other products. Bifidobacteria used in dairy products usually belong to the species: *Bifidobacterium adolescentis; B. animalis* subsp. *animalis; B. animalis* subsp. *lactis; B. bifidum; B. breve; B. infantis; B. longum.*

Because of the wide use of bifidus, ISO, IDF and Sigma-Aldrich have developed new selective Bifidobacteria media. TOS-propionate agar is the ISO/IDF formulation and contains transgalactosylated oligosaccharide as a special growth promoter and for the selectivity of lithium mupirocin. This medium enables the selective enumeration of presumptive bifidobacteria in milk products by using a colony count technique under anaerobic conditions.

Sigma-Aldrich developed their own proprietary formulation called BSM (Bifidus Selective Medium), available as an agar or a broth, as a standard for quality control. This medium contains an additional color reaction which indicates the presence of the bifid shunt metabolism. This medium allows for quick and easy quality control of dairy products made with bifidobacteria and can be used to control the count of bifidobacteria (see **Figure 2**).

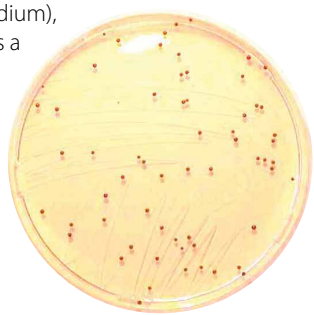

Figure 2: BSM Agar with Bifidobacteria

Cat. No.	Description	Pack Size
90577	Bifido Selective Supplement B	5 vials
88517	BSM Agar	500 g
90273	BSM Broth	500 g
83055	BSM Supplement	5 g
07188	Lithium Mupirocin	1 g 100 mg
69732	Lithium Mupirocin Supplement	10 x 5 mL 10 x 25 mL
43314	TOS-propionate agar	500 g

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